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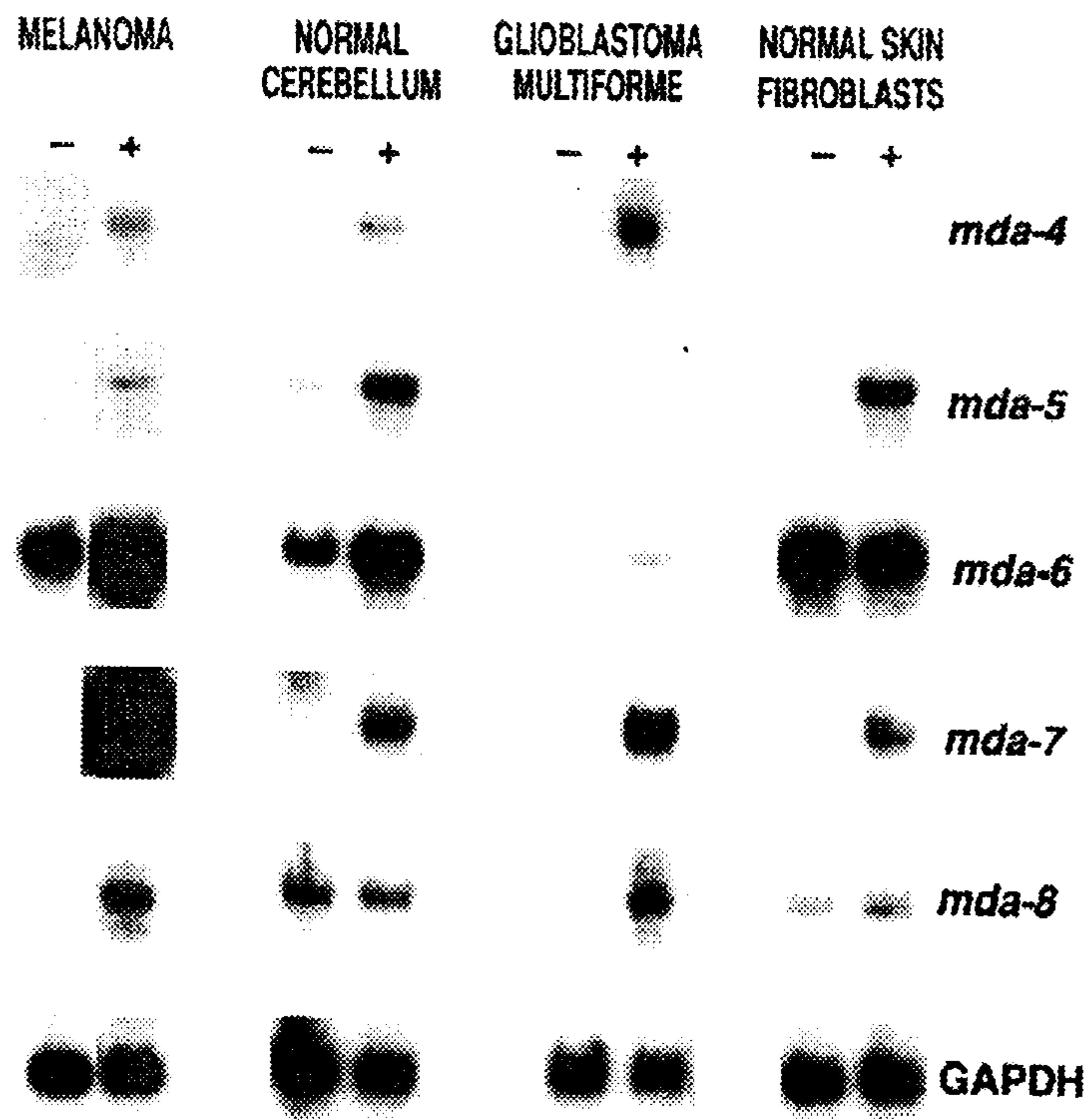
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(54) Titre : COMPOSITIONS COMPRENANT LE GENE 7 ASSOCIE A LA DIFFERENTIATION DES MELANOMES DANS
LE DIAGNOSTIC ET LE TRAITEMENT DU CANCER
(54) Title: COMPOSITIONS OF MELANOMA DIFFERENTIATION ASSOCIATED GENE-7 (MDA-7) IN CANCER
DIAGNOSIS AND TREATMENT



(57) Abrégé/Abstract:

The invention relates to an isolated mda-7 nucleic acid encoding the MDA-7 amino acid sequence of SEQ ID NO:7, the use of the mda-7 gene in the preparation of a pharmaceutical composition and a method of detecting the expression of said gene.



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(54) Title: METHOD FOR GENERATING A SUBTRACTED cDNA LIBRARY AND USES OF THE GENERATED LIBRARY (57) Abstract The invention relates to an isolated <i>mda-7</i> nucleic acid encoding the MDA-7 amino acid sequence of SEQ ID NO:7, the use of the <i>mda-7</i> gene in the preparation of a pharmaceutical composition and a method of detecting the expression of said gene.		

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COMPOSITIONS OF MELANOMA DIFFERENTIATION ASSOCIATED
GENE-7 (mda-7) IN CANCER DIAGNOSIS AND TREATMENT

The invention described herein was supported in part by National Cancer Institute grants CA35675 and CA43208. The United States Government has certain rights in this invention.

Background of the Invention

Throughout this application, various publications are referenced by within parentheses. Full citations for these publications may be found at the end of each series of experiments.

Malignant melanoma is increasing at a rapid rate in North American populations and it is estimated that 1 in 100 children currently born may eventually develop superficial spreading-type melanoma (1). Although readily curable at early stages, surgical and chemotherapeutic intervention are virtually ineffective in preventing metastatic disease and death in patients with advanced states of malignant melanoma (1). These observations emphasize the need for improved therapeutic approaches to more efficaciously treat metastatic

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melanoma. A potentially useful therapeutic modality for this and other malignancies could involve the use of agents capable of inducing an irreversible loss in proliferative capacity in tumor cells without the requirement for direct cytotoxicity, that is, the differentiation therapy of cancer (2-5). In previous studies, applicants have demonstrated that it is possible to reprogram human melanoma cells to undergo terminal cell differentiation with a concomitant loss of proliferative capacity by treatment with the combination of recombinant human fibroblast interferon (IFN- β) plus the antileukemic compound mezerein (MEZ) (6,7). The combination of IFN- β + MEZ induces terminal differentiation in melanoma cells, innately resistant to the antiproliferative effect of either agent alone, and in human melanoma cells selected for resistance to growth suppression induced by IFN- β (6,7). In contrast, treatment with IFN- β or MEZ alone results in the development of specific components of the differentiation program in human melanoma cells, but these agents do not induce most melanoma cells to undergo terminal cell differentiation (6-8).

Terminal differentiation induced by IFN- β plus MEZ in human melanoma cells is associated with an increase in melanin synthesis, changes in cellular morphology (characterized by the production of dendrite-like processes), modifications in cell surface antigenic profile, and an irreversible loss of proliferative capacity (3, 6-10). When used separately, IFN- β and MEZ induce both growth suppression and increased melanin synthesis and MEZ induces the production of dendrite-like processes in specific human melanoma cells (6,8). Trans-retinoic acid (RA) is effective in inducing tyrosinase activity and enhancing melanin synthesis in specific

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human melanoma cultures without altering cell growth, whereas mycophenolic acid (MPA) can induce growth suppression, increased tyrosinase activity and melanin synthesis, and dendrite formation (11). In contrast, the combination of IFN- β + recombinant immune interferon (IFN- γ) results in a synergistic suppression in the growth of human melanoma cells without inducing enhanced melanin synthesis or morphologic changes (10,12). These observations suggest that the various changes induced during the process of differentiation in human melanoma cells, that is, increased tyrosinase activity and melanin synthesis, antigenic changes, dendrite formation, and growth suppression, can occur with and without the induction of terminal cell differentiation.

An unresolved issue is the nature of the gene expression changes that occur in human melanoma cells reversibly committed to differentiation vs. human melanoma cells irreversibly committed to terminal differentiation. This information will be important in defining on a molecular level the critical gene regulatory pathways involved in growth and differentiation in human melanoma cells. To begin to address these questions, applicants have used various experimental protocols that result in either growth suppression without the induction of differentiation-associated properties, a reversible induction of differentiation-associated traits, or terminal cell differentiation in the H0-1 human melanoma cell line. As potential target genes relevant to these processes, applicants have analyzed early growth response, extracellular matrix, extracellular matrix receptor, and interferon-responsive genes. No unique gene expression change was observed solely in H0-1 cells induced to terminally differentiate vs. cultures reversibly growth arrested. However, treatment of H0-1

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cells with IFN- β + MEZ was associated with specific patterns of gene expression changes that were also apparent in H0-1 cells cultured in conditioned medium obtained from terminal-differentiation-inducer-treated H0-1 cells. Exposure to either the terminal differentiation-inducing compounds or -conditioned medium resulted in the enhanced expression of HLA Class I antigen, melanoma growth stimulatory activity (gro-MGSA), interferon-stimulated gene-15 (ISG-15), and fibronectin. These observations support the potential involvement of a type I interferon and a gro/MGSA autocrine loop in the chemical induction of differentiation in H0-1 cells

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Summary of the Invention

This invention provides a method of generating a subtracted cDNA library of a cell comprising: a) generating a cDNA library of the cell; b) isolating double-stranded DNAs from the cDNA library; c) releasing the double-stranded cDNA inserts from the double-stranded DNAs; d) denaturing the isolated double-stranded cDNA inserts; e) hybridizing the denatured double-stranded cDNA inserts with a labelled single-stranded nucleic acid molecules which are to be subtracted from the cDNA library; and f) separating the hybridized labeled single-stranded nucleic acid molecule from the double-stranded cDNA inserts, thereby generating a subtracted cDNA library of a cell. The invention also provides different uses of the generated library.

This invention also provides an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene. This invention further provides different uses of the isolated melanoma differentiation genes.

Brief Description of the Figures

- Fig. 1A-B Effect of continuous exposure to various growth and differentiation modulating agents on the 96-h growth of the human melanoma cell line, H0-1. Cells were seeded at 5×10^4 /35-mm tissue culture plate and 24 h later the medium was changed with the indicated compounds. Cell numbers were determined by Coulter Counter after 96-h growth. Further details can be found in Materials and Methods. The abbreviations and the concentrations of test compounds uses: IFN- β + MEZ (recombinant human fibroblast interferon + mezerein) (2,000 U/ml + 10 ng/ml); MPA + MEZ (mycophenolic acid + MEZ) (3.0 μ M + 10 ng/ml); RA + MEZ (trans-retinoic acid + MEZ) (2.5 μ M + 10 ng/ml); IFN- β + IFN-G (IFN- β + recombinant human gamma interferon) (1,000 U/ml + 1,000 U/ml); MEZ (10 ng/ml); MPA (3.0 μ M); IFN-G (2,000 U/ml); IFN- β (2,000 U/ml); RA (2.5 μ M); and Control (media only).
- Fig. 2 Effect of growth and differentiation modulating agents on steady-state c-jun, jun-B, c-myc, HLA Class I antigen, HLA Class II (DR β), ISG-54, ISG-15, gro/MGSA, and GAPDH mRNA levels in H0-1 cells. Total RNA was isolated 96 h after treatment with the various agents. The concentrations of compounds used were the same as those used for growth studies (see

legend to Figure 1). Ten micrograms of total cytoplasmic RNA was electrophoresed, transferred to nylon filters, and hybridized with the indicated ³²P-labeled gene probes. Further details can be found in Materials and Methods of the first series of experiments.

Fig. 3

Effect on gene expression in H0-1 cells of continuous and transient exposure to agents inducing a reversible commitment to differentiation or terminal cell differentiation. In the left panel [24h (+)], H0-1 cells were treated with IFN- β + MEZ (2,000 U/ml + 10 ng/ml), MPA + MEZ (3.0 μ M + 10 ng/ml), RA + MEZ (2.5 μ M + 10 ng/ml), or MEZ (50) (50 ng/ml) for 24 h. In the right panel [24 h (+) 72 h (-)], H0-1 cells were treated with the same test agents used in the left panel for 24 h, the cells were washed three times in serum-free medium and cells were cultured for an additional 72 h in DMEM-10 in the absence of test compound. Total RNA was isolated, electrophoresed, transferred to nylon filters, and hybridized with the indicated ³²P-labeled gene probes. Further details can be found in Materials and Methods of the first series of experiments.

Fig. 4

Effect of growth and differentiation modulating agents on steady-state α_5 integrin, β_1 integrin, fibronectin, β -

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actin, γ -actin, tenascin, and GAPDH mRNA levels in H0-1 cells. Total RNA was isolated 96 h after treatment with the various agents. The concentrations of compounds used were the same as those used for growth studies (see legend to Figure 1). Ten micrograms of total RNA was electrophoresed, transferred to nylon filters, and hybridized with the indicated ^{32} P-labeled gene probes. Further details can be found in Materials and Methods of the first series of experiments.

Fig. 5

Effect on gene expression in H0-1 cells of continuous and transient exposure to agents, inducing a reversible commitment to differentiation or terminal cell differentiation. Experimental details are described in the legend to Figure 3 and in Materials and Methods of the first series of experiments.

Fig. 6

Effect of conditioned medium from H0-1 cells treated with agents, resulting in a reversible commitment to differentiation or terminal cell differentiation on gene expression in naive H0-1 cells. H0-1 cells were untreated (Control) or treated for 24 h with IFN- β + MEZ (2,000 U/ml + 10 ng/ml), MPA + MEZ (3.0 μ M + 10 ng/ml), RA + MEZ (2.5 μ M + 10 ng/ml), or MEZ (50) (50 ng/ml). The medium was then removed, the cultures were washed three times with serum-free medium, and grown for an

additional 72 h in complete growth medium. The conditioned medium was then collected, and contaminating cells were removed by centrifugation. Conditioned medium was mixed with an equal volume of DMEM-10 (1:2), left panel CONDITIONED MEDIUM (1:2) (24 h), and applied to previously untreated (naive) H0-1 cells for 24 h. Alternatively, conditioned medium was mixed with three parts of DMEM-10 (1:4), right panel CONDITIONED MEDIUM (1:4) (96 h), and applied to previously untreated (naive) H0-1 cells for 96 h. Total RNA was isolated and analyzed by Northern blotting with the DNA probes indicated. Further details can be found in Materials and Methods of the first series of experiments.

Fig. 7

Effect of conditioned medium from H0-1 cells treated with agents, resulting in a reversible commitment to differentiation or terminal cell differentiation on extracellular matrix, extracellular matrix receptor, and cytoskeletal gene expression in naive H0-1 cells. Experimental details are as described in the legend to Figure 6 and in Materials and Methods of the first series of experiments.

Fig. 8

Flowchart for constructing a subtractive differentiation inducer treated human melanoma cell cDNA library. cDNA libraries were constructed from differentiation inducer [IFN- β (2000

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5 units/ml) + MEZ (10 ng/ml)] treated human melanoma (H0-1) cells (*Ind*⁺) and untreated control (*Ind*⁻) H0-1 cells. Using the protocols outlined, an H0-1 IFN- β + MEZ (*Ind*⁺) subtracted cDNA library was constructed.

Fig. 9

10 Determination of the purity of the single-stranded DNA and double-stranded DNA preparations of cDNA libraries. Photograph of a 1% agarose electrophoresis gel stained with ethidium bromide and photographed under illumination with UV light. The single-stranded DNA was prepared from the control (*Ind*⁻) library and double-stranded DNA from IFN- β + MEZ (*Ind*⁺) library. Lane M, λ HindIII molecular weight markers; lane 1, single-stranded DNA; lane 2, single-stranded DNA digested with EcoRI and XhoI; lane 3, double-stranded DNA; lane 4, double-stranded DNA digested with EcoRI and XhoI.

Fig. 10

25 Northern blot analyses of untreated control and IFN- β , MEZ and IFN- β + MEZ treated H0-1 cells probed with cDNA clones isolated from an H0-1 IFN- β + MEZ (*Ind*⁺) subtracted cDNA library. RNA was isolated from cells untreated or treated for 24 hours with IFN- β (2000 units/ml), MEZ (10 ng/ml) or IFN- β + MEZ (2000 units/ml and 10 ng/ml). Ten micrograms of total cellular RNA were separated on a 1% agarose gel, transferred to nylon

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membranes and then hybridized individually with radioactive probes prepared using appropriate inserts from the cDNA clones. The series of cDNAs isolated from inducer treated H0-1 cells have been tentatively called *mda*, melanoma differentiation associated, genes. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a control for uniform loading and expression of the RNA samples.

Fig. 11 Homology of *mda-3* to the cDNA of human macrophage inflammatory protein (GOS19-1). The DNA sequence of *mda-3* was obtained using the Sanger dideoxynucleotide sequencing method. For sequence homology with known genes, the DNA sequence of *mda-3* was compared using the GCG/FASTA program and the GenBank/EMBL database. The top sequence corresponds to the *mda-3* cDNA and the bottom sequence corresponds to GOS19-1.

Fig. 12 Effect of IFN- β and MEZ, alone and in combination on the growth of H0-1 human melanoma cells (see third series of experiments for further details).

Fig. 13 Effect of IFN- β and MEZ, alone and in combination on the morphology of H0-1 human melanoma cells. Cells were treated with 2,000 units/ml of IFN- β , 10 ng/ml MEZ or the combination of agents for 24 h.

Fig. 14 Expression of proliferative sensitive

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proteins (P2Ps) in human melanoma cells treated with growth suppressing and differentiation inducing compounds. The combination of IFN- β + MEZ induces terminal differentiation in F0-1 human melanoma cells, but not in SV40-transformed human melanocytes. Experimental details for determining P2Ps can be found in Minoo et al. (52) and Witte and Scott (53) of the third series of experiments.

Fig. 15

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Northern blot analyses of untreated and treated H0-1 cells probed with cDNA clones isolated from an H0-1 IFN- β + MEZ (*Ind*⁺) subtracted cDNA library. RNA was isolated from cells treated for 96 h with the agents indicated. Concentration of agents were the same as used in Fig. 1. Experimental details can be found in Jiang and Fisher (49) and Jiang et al. (14) of the third series of experiments. IFN- β 2,000 units/ml; IFN- λ 2,000 units/ml; MEZ 10 ng/ml; MPA 3 μ M; RA 2.5 μ M; IFN- β + IFN- γ (1,000 units/ml of each IFN); IFN- β + MEZ (2,000 units/ml + 10 ng/ml); MPA + MEZ (3 μ M + 10 ng/ml); RA + MEZ (2.5 μ M + 10 ng/ml).

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Fig. 16

Expression of mda genes in human melanomas. - = control, + = IFN- β + MEZ (2,000 units/ml + 10 ng/ml).

Fig. 17

Expression of mda genes in normal

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cerebellum, glioblastoma multiforme and normal skin fibroblasts. - = control, + = IFN- β + MEZ (2,000 units/ml + 10 ng/ml).

5 Fig. 18

Expression of mda genes in human colorectal carcinoma (SW613), endometrial adenocarcinoma (HTB113) and prostate carcinoma (LNCaP). - = control, + = IFN- β + MEZ (2,000 units/ml + 10 ng/ml).

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Fig. 19

Effect of various treatment protocols on mda expression in H0-1 cells. IFN- β (2,000 units/ml, 24 hours); MEZ (10 ng/ml, 24 hours); IFN- β + MEZ (2,000 units/ml + 10 ng/ml, 24 hours); phenyl butyrate (PB) (4mM, 24 hours, 4d, 7d); γ Rad (γ irradiation) 3 gray, 24 hours, Act D (actinomycin D) (5 μ g/ml, 2 hour $\rightarrow$ 24 hours assay); Adriamycin* (Adr) (0.1 μ g/ml, 24 hours); Vincristine (Vin) (0.1 μ g/ml, 24 hours); cis-plt (cis-platinum) (0.1 μ g/ml, 24 hours); TNF- α (tumor necrosis factor - α) (100 units/ml, 24 hours); UV (10 joules/mm², 2, 14 and 24 hours assay); VP-16 (5 μ g/ml, 24 hours); IFN- α (2,000 units/ml, 24 hours); and IFN- α + MEZ (2,000 units/ml + 10 ng/ml, 24 hours).

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Fig. 20.

Stages in the development of metastatic melanoma.

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Fig. 21A-E.

Effect of recombinant human IFN- β and mezerein (MEZ) used alone and in combination on the morphology of H0-1 and

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Fig. 22

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B0-2 human melanoma cells (X150). (A) Control H0-1 cells 24 h postplating. (B) H0-1 cells exposed to 2,000 units/ml IFN- β for 24 h. (C) H0-1 cells exposed to 10 ng/ml MEZ for 24 h. (D) H0-1 cells exposed to 2,000 units/ml IFN- β and 10 ng/ml MEZ for 24 h. (E) Control B0-2 cells 24 h postplating. (F) B0-2 cells exposed to 2,000 units/ml IFN- β for 24 h. (G) B0-2 cells exposed to 10 ng/ml MEZ for 24 h. (H) B0-2 cells exposed to 2000 units/ml IFN- β and 10 ng/ml MEZ for 24 h. Data from reference [18] of the fifth series of experiments.

Components of the differentiation process in H0-1 human melanoma cells. Abbreviations: IFN- β : recombinant human fibroblast interferon; IFN- γ : recombinant immune interferon; MPA: mycophenolic acid; MEZ: mezerein; Trans RA: trans retinoic acid. Relative growth suppression: 4+ = ~80% reduction in growth in comparison with untreated control cultures; 3+ = ~50 to 60% reduction in growth in comparison with untreated control cultures; 2+ = ~40% reduction in growth in comparison with untreated control cultures; 1+ = ~30% reduction in growth comparison with untreated control cultures.

Fig. 23A-B

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cDNA and predicted amino acid sequence of *mda-6*. The predicted translation begins at nucleotide 95 and ends at nucleotide 589. Accession number U09579 (GeneBank).

Fig. 24

Cellular changes mediating enhanced expression of certain *mda* genes and differential expression of particular *mda* genes during tumor progression and in normal versus tumor-derived cell types. Specific *mda* genes have been identified that display enhanced expression during treatment with agents that induce growth suppression, DNA damage (including chemotherapeutic agents that function by different mechanisms) and/or terminal differentiation. Additional *mda* genes have also been shown to display differential expression as a function of tumor progression and in matched sets of normal versus tumor-derived human cells.

Fig. 25A-B

Induction of *mda-6* (WAF1/CIP1/SDI1) expression in HL-60 cells by TPA and RA. Total cytoplasmic RNA was isolated from HL-60 cells treated with TPA (3 nM) for .25, .5, 1, 2, 4 or 6 d and from HL-60 cells treated with RA (1 μ M) for .5, 1, 2, 4 or 6 d. A 10- μ g aliquot of RNA was run on a 1.0% agarose gel and transferred to a nylon filter. Blots were hybridized with a multi-prime ^{32} P-labeled *mda-6* gene probe. Filters were stripped and rehybridized with a multiprime ^{32} P-labeled GAPDH probe.

Fig. 26A-C

Early induction of *mda-6* (WAF1/CIP1/SDI1) expression in HL-60 and TPA-resistant HL-60 (HL-525) cells treated with TPA, RA and

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Vit D3. Cells were treated with TPA (3 nM), RA (1 μ M) or Vit D3 (400 nM) for 1, 2, 3, 6 or 12 h. RNA was isolated and analyzed by RT-PCR using appropriate *mda-6* or GAPDH specific primers as described in the Materials and methods.

Fig. 27A-C

Induction of *mda-6* (WAF1/CIP1/SDI1) expression in HL-60 and TPA-resistant HL-60 (HL-525) cells after extended incubation with TPA, RA and Vit D3. Cells were treated with TPA (3 nM), RA (1 μ M) or Vit D3 (400 nM) for .5, 1, 2, 4 or 6 d. RNA was isolated and analyzed by RT-PCR using appropriate *mda-6* or GAPDH specific primers as described in the Materials and methods.

Fig. 28

Induction of the MDA-6 (WAF1/CIP1/SDI1) encoded protein p21 in HL-60 cells treated with TPA, DMSO and RA. Lysates from untreated HL-60 (control) and HL-60 cells treated with TPA (3 nM), DMSO (1%) or RA (1 μ M) for 12, 24, 48 and 72 h were immunoprecipitated with WAF1/CIP1 (MDA-6) polyclonal antibody or actin monoclonal antibody as described in Materials and methods. The size of the MDA-6 protein is 21 kDa and the size of the Actin protein is 42 kDa.

Fig. 29A-B

Effect of CHX on the induction of *mda-6* in HL-60 and TPA-resistant HL-60 (HL-525) cells. Cells were treated with CHX (10 μ g/ml) for 1, 3, 6 or 10 h or with CHX (10

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5 μ g/ml) plus TPA (3 nM) for 1+, 3+, 6+ or 10+ h. RNA was isolated and analyzed by RT-PCR using appropriate *mda-6* or GAPDH specific primers as described in the Materials and methods in the sixth series of experiments.

Fig. 30

10 Open-reading frame of *mda-6*. Predicted translation of the *mda-6* cDNA begins at nucleotide 95 and ends a nucleotide 589. Accession number U09579 (Genbank). *mda-6* encodes a 164 amino acid protein with an M of 21,000 that is identical to the cyclin dependent kinase inhibitor, p21.

Fig. 31A-E

15 Induction of *mda-6* (p21) expression in H0-1 human melanoma cells as a function of differentiation and growth suppression. H0-1 cells were untreated (control) or treated for 24 h (panel A), 96 h (panel B) or treated for 24 h followed by growth for 72 h in the absence of inducer (panel C) with IFN- β (2000 units/ml), MEZ (10 ng/ml) or IFN- β + MEZ (2000 units/ml + 10 ng/ml). In panel D, H0-1 cells were grown for the indicated time in medium containing 5% fetal bovine serum (Control) or without fetal bovine serum (DMEM-0). In panel E, high-density H0-1 cells were incubated in DMEM-0 for the times indicated. RNA isolation, Northern blotting and hybridization with *mda-6* and GAPDH was performed as described (Jiang & Fisher, 1993; Jiang et al., 1993).

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Fig. 32

Effect of IFN- β + MEZ on *mda-6* (p21) expression in human melanoma cells and an SV40-transformed human melanocyte culture. High-density melanoma cells (H0-1, F0-1, L0-1, SH-1, WM239 and WM278) and logarithmically growing low-density SV40-transformed human melanocytes (SV516-SV) were treated with IFN- β (2000 units/ml) + MEZ (10 ng/ml) for 24 hr, total RNA was isolated and analyzed by Northern blotting (Jiang & Fisher, 1993; Jiang et al., 1993). Filters were probed with *mda-6* then stripped and probed with GAPDH as described (Jiang & Fisher, 1993; Jiang et al., 1993).

Fig. 33

Effect of 24 h treatment of H0-1 cells with IFN- β , MEZ and IFN- β + MEZ on p53 and p21 levels. Lysates from untreated (control) and H0-1 cells treated with IFN- β (2000 units/ml), MEZ (10 ng/ml) or IFN- β + MEZ (2000 units/ml + 10 ng/ml) for 24 h were immunoprecipitated with p53 monoclonal antibody Ab1 (PAb421), p21 (WAF1/CIP1) polyclonal antibody or an actin monoclonal antibody as described in Materials and methods in the seventh series of experiments.

Fig. 34

Expression of p53 and p21 as a function of growth arrest and terminal differentiation in H0-1 cells. Lysates from untreated (control) and H0-1 cells treated with IFN- β (2000 units/ml), MEZ (10 ng/ml) or IFN- β + MEZ (2000 units/ml + 10 ng/ml) for 48,

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72 and 96 h were immunoprecipitated with p53 monoclonal antibody Ab1 (PAb421), p21 (WAF1/CIP1) polyclonal antibody or an actin monoclonal antibody as described in Materials and methods in the seventh series of experiments.

Fig. 35

Relative expression of *mda-6* during melanoma progression. The level of *mda-6* relative to GAPDH was determined by quantitative (comparative) RT-PCR in actively growing cells. RGP: radial growth phase primary melanoma. VGP: vertical growth phase primary melanoma. Results represent average for: 5 melanocyte cultures, one dysplastic nevus culture, one SV40-transformed immortalized melanocyte culture, one RGP and four early VGP primary melanomas and six metastatic melanomas.

Fig. 36

Expression of *mda-6* (p21) as a function of Matrigel-mediated progression in primary human melanomas. The level of *mda-6* and GAPDH was determined by RT-PCR for the indicated actively proliferating cell lines. P1 and P2 refers to the first and second passage, respectively, through nude mice of RGP or early VGP melanoma cells in combination with Matrigel.*

Fig. 37

Northern blot analysis of *mda-6* (p21) expression in C8161 human melanoma and chromosome 6 containing C8161 human melanoma subclones. Levels of *mda-6* and

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GAPDH mRNAs were determined in actively growing untreated cells (-) and cells treated for 96 hr with IFN- β + MEZ (1000 units/ml + 10 ng/ml) as described (Jiang & Fisher, 1993; Jiang et al., 1994a).

Fig. 38A-B

Nucleotide sequence and deduced encoded amino acid sequence of the *mda-7* cDNA. The hydrophobic putative transmembrane domain and three potential N-glycosylation residues are underlined; the 3' untranslated sequence contains three putative instability motifs ATTA (underlined) and two consensus signals AATAAA for poly(A) addition (underlined).

Fig. 39

Properties and structure of the *mda-7* encoded gene product as predicted by GCG/Plotstructure* program.

Fig. 40

Hydrophobic analysis and structural predictions of the *mda-7* encoded gene product by GCG/Peppplot* program. A potential transmembrane domain is predicted using this program.

Fig. 41

Structure of *mda-7* encoded gene produce as predicted by the Chou-Fosman method.

Fig. 42

Relative expression of *mda-7* during melanoma progression. The level of *mda-7* relative to GAPDH was determined quantitative (comparative) RT-PCR in actively growing cells. The cell lines examined were (1) normal melanocytes; (2)

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primary melanoma cells, including RGP and VGP cells; and (3) metastatic melanoma cells.

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Expression of *mda-6* and *mda-7* as a function of aging and senescence in human fibroblast cells. IMR90 cells analyzed at late passage (OLD) versus early passage (YOUNG). IDH4 cells are IMR90 cells immortalized by an SV40 T-antigen (transcriptionally controlled by a dexamethasone (DEX) inducible mouse mammary tumor virus promoter). Growth of IDH4 cells in DEX results in active growth and expression of an immortalized phenotype (a YOUNG IMR90 phenotype). In contrast, removal of DEX results in a shutdown of the T-antigen and loss of proliferative capacity and senescence (SENESCENT). RNA was extracted from the four cell lines and analyzed by reverse transcription-polymerase chain reaction (RT-PCR) using primer sequences specific for *mda-6*, *mda-7* or GAPDH. As a control for expression of these genes, RNA from H0-1 human melanoma cells treated with IFN- β + MEZ (2000 units/ml + 10 ng/ml) for 96 hr was used. H0-1 cells under these conditions are irreversibly growth arrested and terminally differentiated.

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Detailed Description of the Invention

Throughout this application, the following standard abbreviations are used to indicate specific nucleotides:

5 C=cytosine A=adenosine
 T=thymidine G=guanosine

10 This invention provides a method of generating a
 subtracted cDNA library of a cell comprising: a)
 generating a cDNA library of the cell; b) isolating
 double-stranded DNAs from the cDNA library; c) releasing
 the double-stranded cDNA inserts from the double-stranded
 DNAs; d) denaturing the isolated double-stranded cDNA
15 inserts; e) hybridizing the denatured double-stranded
 cDNA inserts with a labelled single-stranded nucleic acid
 molecules which are to be subtracted from the cDNA
 library; and f) separating the hybridized labeled single-
 stranded nucleic acid molecule from the double-stranded
20 cDNA inserts, thereby generating a subtracted cDNA
 library of a cell.

In an embodiment, the cDNA library of the cell is a λ ZAP^{*}
cDNA library.

25 This invention provides the above-described method,
 wherein the releasing of the double-stranded cDNA is
 performed by digestion with appropriate restriction
 enzymes.

30 This invention also provides the above-method of
 generating a subtracted cDNA library of a cell, wherein
 the denaturing of step d) is by boiling.

35 In an embodiment, the single-stranded nucleic acid
 molecules are DNAs. In a further embodiment, the single-

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stranded nucleic acid molecules are labelled with biotin.
In a still further embodiment, the single-stranded
nucleic acid molecules are labelled with biotin, wherein
the separating of step f) is performed by extraction with
5 streptavidin-phenol: Chloroform.

Other methods for labelling single-stranded nucleic acid
molecules are well-known in the art.

10 This invention further provides the above-described
methods of generating a subtracted cDNA library of a
cell, wherein the single-stranded nucleic acid molecules
are from another cDNA library. In another embodiment,
this cDNA library is a λ ZAP cDNA library.

15 In another embodiment, the single-stranded nucleic acid
molecules are from another cDNA library, wherein the cDNA
library is a λ ZAP cDNA library, wherein the cell is an
HO-1 melanoma cell treated with IFN- β and MEZ.

20 In another embodiment, wherein the cDNA library is a λ ZAP
cDNA library, the cell is an HO-1 melanoma cell treated
with IFN- β and MEZ and the single-stranded nucleic acid
molecules are from another cDNA library of a HO-1
25 melanoma cell.

In still another embodiment of the above-described
methods of generating a subtracted cDNA library of a
cell, wherein the cDNA library is a λ ZAP cDNA library,
30 wherein the cell is terminally differentiated and the
single-stranded nucleic acid molecules are from another
cDNA library of an undifferentiated cell.

In still another embodiment of the above-described
35 methods of generating a subtracted cDNA library of a

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cell, wherein the cDNA library is a λ ZAP cDNA library, wherein the cell is undifferentiated and the single-stranded nucleic acid molecules are from another cDNA library of a terminally differentiated cell.

5 In one embodiment of the above-described method of generating a subtracted cDNA library of a cell, the cell is selected from a group consisting essentially of neuroblastoma cell, glioblastoma multiforme cell, myeloid
10 leukemic cell, breast carcinoma cell, colon carcinoma cell, endometrial carcinoma cell, lung carcinoma cell, ovarian carcinoma cell and prostate carcinoma cell.

15 In another embodiment of the above-described method of generating a subtracted cDNA library of a cell, the cell is induced to undergo reversible growth arrest or DNA damage and the single-stranded nucleic acid molecules are from another cDNA library of an uninduced cell.

20 In still another embodiment of the above-described method of generating a subtracted cDNA library of a cell, the cell is at one developmental stage and the single-stranded nucleic acid molecules are from another cDNA library from the cell at different developmental stage.

25 In still another embodiment of the above-described method of generating a subtracted cDNA library of a cell, the cell is cancerous and the single-stranded nucleic acid molecules are from another cDNA library from a normal
30 cell.

In another embodiment, the cell is from the breast, brain, meninges, spinal cord, colon, endometrium, lung, prostate and ovary.

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This invention also provides a method of generating a subtracted cDNA library of a cell comprising: a) generating a cDNA library of the cell; b) isolating double-stranded DNAs from the cDNA library; c) releasing the double-stranded cDNA inserts from the double-stranded DNAs; d) denaturing the isolated double-stranded cDNA inserts; e) hybridizing the denatured double-stranded cDNA inserts with a labelled single-stranded nucleic acid molecules which are to be subtracted from the cDNA library; and f) separating the hybridized labeled single-stranded nucleic acid molecule from the double-stranded cDNA inserts, thereby generating a subtracted cDNA library of a cell, wherein the single-stranded nucleic acid molecules are from another cDNA library, wherein the cDNA library is a λ ZAP cDNA library, further comprising introducing the subtracted library into host cells.

This invention provides a subtracted library generated by the method generating a subtracted cDNA library of a cell comprising: a) generating a cDNA library of the cell; b) isolating double-stranded DNAs from the cDNA library; c) releasing the double-stranded cDNA inserts from the double-stranded DNAs; d) denaturing the isolated double-stranded cDNA inserts; e) hybridizing the denatured double-stranded cDNA inserts with a labelled single-stranded nucleic acid molecules which are to be subtracted from the cDNA library; and f) separating the hybridized labeled single-stranded nucleic acid molecule from the double-stranded cDNA inserts, thereby generating a subtracted cDNA library of a cell.

This invention provides a subtracted library generated by the method of generating a subtracted cDNA library of a cell comprising: a) generating a cDNA library of the cell; b) isolating double-stranded DNAs from the cDNA

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library; c) releasing the double-stranded cDNA inserts from the double-stranded DNAs; d) denaturing the isolated double-stranded cDNA inserts; e) hybridizing the denatured double-stranded cDNA inserts with a labelled single-stranded nucleic acid molecules which are to be subtracted from the cDNA library; and f) separating the hybridized labeled single-stranded nucleic acid molecule from the double-stranded cDNA inserts, thereby generating a subtracted cDNA library of a cell, wherein the single-stranded nucleic acid molecules are from another cDNA library, wherein the cDNA library is a λ ZAP cDNA library, wherein the cell is an H0-1 melanoma cell treated with IFN- β and MEZ, wherein the single-stranded nucleic acid molecules are from another cDNA library of a H0-1 melanoma cell.

This invention provides a method of identifying a melanoma differentiation associated gene comprising: a) generating probes from clones of the above-described subtracted library; and b) hybridizing the probe with total RNAs or mRNAs from H0-1 cells treated with IFN- β and MEZ and total RNAs or mRNAs from untreated H0-1 cells, hybridization of the probe with the mRNAs from the treated H0-1 cell but no or reduced hybridization with the total RNAs or mRNAs from untreated cells indicating that the clone from which the probe is generated carries a melanoma differentiation associated gene.

This invention further provides a melanoma differentiation associated gene identified by the above method.

This invention provides a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of mda-4 gene.

5 This invention further provides a method of detecting the expression of *mda-4* gene in a cell comprising: a) isolating the nucleic acids in the cell; b) hybridizing the isolated nucleic acids with the nucleic acid molecule capable of specifically hybridizing with a sequence of *mda-4* gene under conditions permitting hybrid formation; and c) detecting hybrids formed, the detection of the hybrids indicating expression of *mda-4* gene in the cell.

10 This invention also provides a method to indicate the tissue lineage of a cell comprising detecting the expression of *mda-4* gene using the above-described method, the expression of *mda-4* gene indicating that the tissue lineage of the cell is neuroectodermal.

15 This invention also provides a method for distinguishing a fibroblast or epithelial cell from a melanoma or central nervous system cell comprising detecting the expression of *mda-4* gene using the above-described method, the expression of *mda-4* indicating that the cell is a melanoma cell or a central nervous system lineage cell.

20 This invention provides a method to monitor the response to DNA damage induced by gamma irradiation and UV irradiation of a cell comprising hybridizing the nucleic acid molecule capable of specifically hybridizing with a sequence of *mda-4* gene, hybridization of the nucleic acids from the cell indicating that there is a response to the DNA damage of the cell.

25 This invention provides a method of monitoring a response to treatment with chemotherapeutic agents which work in a similar manner as cis-platinum in a cell comprising hybridizing the nucleic acids from the cell with the

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nucleic acid molecule capable of specifically hybridizing with a sequence of *mda-4* gene, hybridization of the nucleic acids from the cell responds to the treatment with the chemotherapeutic agents.

5 This invention further provides a method for detecting types I or II interferons in a sample comprising: a) incubating the sample with the 5' regulatory element of the *mda-4* gene under conditions permitting binding of
10 types I and II interferons transcriptional regulatory proteins to the regulatory elements; and b) detecting the binding of types I or II interferons transcriptional regulatory proteins to the regulatory elements, the binding indicating the presence of type I or II
15 interferons in the sample.

As used herein, the term "sample" is broadly defined. It includes, but not limited to bodily fluids such as urine, saliva, blood and other clinical samples.

20 Transcriptional regulatory proteins which are responsive to type I or II interferons are well-known in the art.

In an embodiment, the target cell is a eukaryotic cell.
25 In a separate embodiment, the 5' regulatory element is linked to the native *mda-4* gene and the detection of binding is by examination of the elevated expression of the *mda-4* gene. In an embodiment, the 5' regulatory element is linked to a marker gene. In a further
30 embodiment, the marker gene is β -galactosidase or CAT.

This invention provides an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-1*. In an embodiment, the
35 nucleic acid is a cDNA. In another embodiment, the

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nucleic acid is genomic DNA.

In a separate embodiment, the *mda-1* gene is coding for a human protein.

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This invention provides an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-2*. In an embodiment, the nucleic acid is a cDNA. In another embodiment, the

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In a separate embodiment, the *mda-2* gene is coding for a human protein.

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This invention provides an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-4*. In an embodiment, the nucleic acid is a cDNA. In another embodiment, the nucleic acid is genomic DNA.

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In a separate embodiment, the *mda-4* gene is coding for a human protein.

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This invention provides an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-5*. In an embodiment, the nucleic acid is a cDNA. In another embodiment, the nucleic acid is genomic DNA.

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In a separate embodiment, the *mda-5* gene is coding for a human protein.

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This invention provides a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid molecule, *mda-5*.

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5 This invention provides a method of detecting the expression of *mda-5* gene in a cell comprising: a) isolating the nucleic acids in the cell; b) hybridizing the isolated nucleic acids with the nucleic acid molecules of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid molecule *mda-5* under conditions permitting hybrids formation; and c) detecting hybrids formed, the detection of the hybrids formed indicating the expression of *mda-5* gene in the cell.

15 This invention provides a method for distinguishing a normal neuroectodermal cell from a malignant neuroectodermal cell comprising detecting the expression of *mda-5* gene using the method of detecting the expression of *mda-5* gene in a cell comprising: a) isolating the nucleic acids in the cell; b) hybridizing the isolated nucleic acids with the nucleic acid molecules of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid molecule *mda-5* under conditions permitting hybrids formation; and c) detecting hybrids formed, the detection of the hybrids formed indicating the expression of *mda-5* gene in the cell, the expression of *mda-5* gene indicating that the cell is normal neuroectodermal cell.

25 This invention provides a method for detecting types I or II interferons in a sample comprising: a) incubating the sample with the 5' regulatory element of the *mda-5* gene under conditions permitting binding of types I and II interferons transcriptional regulatory proteins to the regulatory elements; and b) detecting the binding of the types I or II interferons transcriptional regulatory proteins to the regulatory elements, the binding indicating the presence of type I or type II interferons

in the sample.

5 In an embodiment, the cell is a eukaryotic cell. In another embodiment, the 5' regulatory element is linked to the native *mda-5* gene and the detection of binding is by the examination of the elevated expression of *mda-5* gene.

10 In a separate embodiment, the 5' regulatory element is linked to a marker gene. In a further embodiment, the marker gene is β -galactosidase, luciferase or CAT.

15 This invention provides a method for identifying a compound capable of inducing terminal differentiation in human melanoma cells comprising: a) incubating the human melanoma cells with an appropriate concentration of the compound; and b) detecting the expression of *mda-5* using the above-described method, the expression of *mda-5* gene indicating that the compound is capable of inducing terminal differentiation in human melanoma cells.

20 This invention provides an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-6*. In an embodiment, the nucleic acid is a cDNA. In another embodiment, the nucleic acid is genomic DNA.

25 In a separate embodiment, the *mda-6* gene is coding for a human protein.

30 This invention provides a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid molecule, an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-*

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This invention provides a method of detecting the expression of *mda-6* gene in a cell comprising: a) isolating the nucleic acids in the cell; b) hybridizing the isolated nucleic acids with the nucleic acid molecules of a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid molecule, an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-6* under conditions permitting hybrids formation; and c) detecting hybrids formed, the detection of the hybrids formed indicating the expression of *mda-6* gene in the cell.

This invention provides a method for distinguishing a normal neuroectodermal cell from a malignant neuroectodermal cell comprising detecting the expression of *mda-6* gene using the method of detecting the expression of *mda-6* gene in a cell comprising: a) isolating the nucleic acids in the cell; b) hybridizing the isolated nucleic acids with the nucleic acid molecules capable of recognizing the *mda-6* gene under conditions permitting hybrids formation; and c) detecting hybrids formed, the detection of the hybrids formed indicating the expression of *mda-6* gene in the cell, the expression of *mda-6* gene indicating that the cell is normal neuroectodermal cell.

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The invention also provides a method for distinguishing an adenocarcinoma cell from a carcinoma cell comprising detecting the expression of *mda-6* gene using the above-described method, the expression of *mda-6* gene indicating that the cell is a carcinoma cell.

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5 The invention further provides a method for monitoring the response of a cell to an anticancer agent such as actinomycin-D or adriamycin comprising detecting the expression of *mda-6* gene using the above-described method, the expression of *mda-6* gene indicating that the cell responds to the anticancer agent.

10 This invention provides a method for monitoring response to topoisomerase inhibitor by a cell comprising detecting the expression of *mda-6* using the above-described method of detecting the expression of *mda-6* gene in a cell, the expression of *mda-6* indicating that the cell responds to the topoisomerase inhibitor.

15 This invention provides a method for identifying a compound capable of inducing terminal differentiation in human melanoma cells comprising: a) incubating the human melanoma cells with an appropriate concentration of the compound; and b) detecting the expression of *mda-6* in a
20 cell using the above-described method, the expression of *mda-6* gene indicating that the compound is capable of inducing terminal differentiation in human melanoma cells.

25 This invention provides a method for identifying a compound capable of inducing terminal differentiation in human leukemia cells comprising a) incubating the human leukemia cells with an appropriate concentration of the compound; and b) detecting the expression of *mda-6* using
30 the above-described method, the expression of *mda-6* gene indicating that the compound is capable of inducing terminal differentiation in human leukemia cells.

35 This invention also provides a method for identifying a compound capable of inducing terminal differentiation in

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human lymphoma cells comprising: a) incubating the human lymphoma cells with an appropriate concentration of the compound; and b) detecting the expression of mda-6 using the above-described method, the expression of mda-6 gene indicating that the compound is capable of inducing terminal differentiation in human lymphoma cells.

This invention provides a method for identifying a compound capable of inducing terminal differentiation in human neuroblastoma cells comprising: a) incubating the human neuroblastoma cells with an appropriate concentration of the compound; and b) detecting the expression of mda-6 using the above-described method, the expression of mda-6 gene indicating that the compound is capable of inducing terminal differentiation in human neuroblastoma cells.

This invention also provides a method for identifying a compound capable of inducing terminal differentiation in human glioblastoma multiforme cells comprising: a) incubating the human glioblastoma cells with an appropriate concentration of the compound; and b) detecting the expression of mda-6 using the above-described method, the expression of mda-6 gene indicating that the compound is capable of inducing terminal differentiation in human glioblastoma multiforme cells.

This invention also provides a method for distinguishing an early stage from a more progressed human melanoma cell comprising detecting the expression of mda-6 gene using the above-described method, the expression of mda-6 gene indicating that the cell is a less progressed human melanoma cell.

This invention provides a method for reversing the

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5 malignant phenotype of cells comprising: (a) linking the *mda-6* gene to a regulatory element such that the expression of the *mda-6* gene is under the control of the regulatory element; and (b) introducing the linked *mda-6* gene into the malignant cells for the expression of the *mda-6* gene, thereby reversing the malignant phenotype of cells.

10 This invention also provides a method for reversing the malignant phenotype of cells comprising: (a) linking the *mda-6* gene to a regulatory element such that the expression of the *mda-6* gene is under the control of the regulatory element; (b) introducing the linked *mda-6* gene into the malignant cells; and (c) placing the cells from
15 step (b) in appropriate conditions to express the *mda-6* gene such that the expression of the *mda-6* gene will reverse the transforming phenotype of the malignant cells.

20 This invention also provides a method of reversing the phenotype of malignant cells in a subject comprising:
(a) linking the *mda-6* gene to a regulatory element such that the expression of the *mda-6* gene is under the control of the regulatory element; (b) introducing the
25 linked *mda-6* gene into the malignant cells for the expression of the *mda-6* gene, thereby reversing the phenotype of the malignant cells.

30 This invention also provides a method of reversing the phenotype of malignant cells in a subject comprising:
(a) linking the *mda-6* gene to a regulatory element such that the expression of the *mda-6* gene is under the control of the regulatory element; (b) introducing the linked *mda-6* gene into the malignant cells of the
35 subject; and (c) inducing the expression of the *mda-6*

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gene which will reverse the transforming properties of the cells, thereby reversing the phenotype of the malignant cells in the subject.

5 This invention also provides a method of inducing growth suppression in tumorigenic and metastatic cells comprising: (a) linking the *mda-6* gene to a regulatory element such that the expression of the *mda-6* gene is under the control of the regulatory element; (b)
10 introducing the linked *mda-6* gene into the tumorigenic and metastatic cells; and (c) inducing the expression of the *mda-6* gene, thereby inducing growth suppression in tumorigenic and metastatic cells.

15 This invention also provides a method of inducing terminal differentiation in tumorigenic and metastatic cells comprising: (a) linking the *mda-6* gene to a regulatory element such that the expression of the *mda-6* gene is under the control of the regulatory element; (b)
20 introducing the linked *mda-6* gene into the tumorigenic and metastatic cells; and (c) inducing the expression of the *mda-6* gene, thereby inducing terminal differentiation in tumorigenic and metastatic cells.

25 In an embodiment, the cell is a melanoma, leukemia, lymphoma, neuroblastoma, glioblastoma or carcinoma cell.

In a separate embodiment, the regulatory element is a promoter. In a further embodiment, the promoter is a
30 tissue-specific promoter. In another embodiment, the promoter is an inducible promoter.

The linked *mda-6* gene may be introduced into the cells by naked DNA technology, retroviral vectors, antibody-coated
35 liposomes, mechanical or electrical means. These

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technologies are known in the art.

5 This invention provides a method of determining the stage of a melanoma comprising: (a) obtaining appropriate amount of cells from the melanoma; (b) measuring the expression level of the *mda-6* gene in the cells; and (b) comparing the expression level with predetermined standards of normal and melanoma cells in different stages, thereby determining the stage of a melanoma.

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In an embodiment, the expression is measured by the antibodies against the *mda-6* protein. In another embodiment, the expression is measured by in situ hybridization.

15

20 This invention also provides a method for indicating the effectiveness of a treatment against cancer comprising measuring the expression level of *mda-6* gene in the cells of the cancer, the increase of the expression level indicating the effectiveness of the treatment. In an embodiment, the cancer is melanoma. In another embodiment, the cancer is leukemia. In a separate embodiment, the cancer is lymphoma. In another embodiment, the cancer is neuroblastoma. In a further embodiment, the cancer is a glioblastoma multiforme tumor. In a still further embodiment, the cancer is a carcinoma.

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30 This invention provides an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-7*. In an embodiment, the nucleic acid is a cDNA. In another embodiment, the nucleic acid is genomic DNA.

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35 In a separate embodiment, the *mda-7* gene is coding for a

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human protein.

This invention provides an isolated nucleic acid molecule of a cDNA, wherein the protein is a human protein.

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This invention provides a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid molecule of *mda-7*.

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This invention provides a method of detecting the expression of *mda-7* gene in a cell comprising: a) isolating the nucleic acids in the cell; b) hybridizing the isolated nucleic acids with the nucleic acid molecules of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid molecule of an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-7* under conditions permitting hybrids formation; and c) detecting hybrids formed, the detection of the hybrids formed indicating the expression of *mda-7* gene in the cell.

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This invention provides a method for determining whether a cell is a melanoma cell or a carcinoma cell comprising detecting the expression of *mda-7* gene using the above-described method of detecting the expression of *mda-7* gene in a cell, the expression of *mda-7* gene indicating that the cell is a melanoma cell.

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The invention further provides a method for distinguishing a melanocyte or early stage melanoma cell from an advanced metastatic melanoma cell comprising detecting the expression of *mda-7* gene using the above-described method, the expression of *mda-7* gene indicating that the cell is a melanocyte or early stage melanoma

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cell.

5 This invention provides a method for distinguishing a normal neuroectodermal cell from a malignant neuroectodermal cell comprising detecting the expression of *mda-7* gene using the above-described method of detecting the expression of *mda-7* gene in a cell, the expression of *mda-7* gene indicating that the tissue lineage of the cell is normal neuroectodermal cell.

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The invention also provides a method for distinguishing a fibroblast from an epithelial cell comprising detecting the expression of *mda-7* gene using the above-described method, the expression of *mda-7* gene indicating that the cell is a fibroblast.

15

20 This invention provides a method for identifying a compound capable of inducing growth suppression in human melanoma cells comprising: a) incubating appropriate concentration of the human melanoma cells with an appropriate concentration of the compound; and b) detecting the expression of *mda-7* using the above-described method, the expression of *mda-7* gene indicating that the compound is capable of inducing growth suppression in human melanoma cells.

25

30 The invention further provides a method for monitoring the response of a cell to an anticancer agent such as adriamycin or vincristine comprising detecting the expression of *mda-7* gene using the above-described method, the expression of *mda-7* gene indicating that the cell responds to the anticancer agent.

35 The invention also provides a method for monitoring the response of a cell to DNA damage induced by UV

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irradiation comprising detecting the expression of *mda-7* gene using the above-described method, the expression of *mda-7* gene indicating that the cell responds to the DNA damage.

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This invention provides a method for reversing the malignant phenotype of cells comprising: (a) linking the *mda-7* gene to a regulatory element such that the expression of the *mda-7* gene is under the control of the regulatory element; and (b) introducing the linked *mda-7* gene into the malignant cells for the expression of the *mda-7* gene, thereby reversing the malignant phenotype of cells.

15 This invention also provides a method for reversing the malignant phenotype of cells comprising: (a) linking the *mda-7* gene to a regulatory element such that the expression of the *mda-7* gene is under the control of the regulatory element; (b) introducing the linked *mda-7* gene into the malignant cells; and (c) placing the cells from step (b) in appropriate conditions to express the *mda-7* gene such that the expression of the *mda-7* gene will reverse the transforming phenotype of the malignant cells.

25

This invention also provides a method for reversing the phenotype of malignant cells in a subject comprising: (a) linking the *mda-7* gene to a regulatory element such that the expression of the *mda-7* gene is under the control of the regulatory element; and (b) introducing the linked *mda-7* gene into the malignant cells for the expression of the *mda-7* gene, thereby reversing the phenotype of the malignant cells.

35 This invention also provides a method to reversing the

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phenotype of malignant cells in a subject comprising:

(a) linking the *mda-7* gene to a regulatory element such that the expression of the *mda-7* gene is under the control of the regulatory element; (b) introducing the linked *mda-7* gene into the malignant cells of the subject; and (c) inducing the expression of the *mda-7* gene which will reverse the transforming properties of the cells, thereby reversing the phenotype of the malignant cells in the subject.

This invention also provides a method of inducing growth suppression in tumorigenic and metastatic cells comprising: (a) linking the *mda-7* gene to a regulatory element such that the expression of the *mda-7* gene is under the control of the regulatory element; (b) introducing the linked *mda-7* gene into the tumorigenic and metastatic cells; and (c) inducing the expression of the *mda-7* gene, thereby inducing growth suppression in tumorigenic and metastatic cells.

This invention also provides a method of inducing terminal differentiation in tumorigenic and metastatic cells comprising: (a) linking the *mda-7* gene to a regulatory element such that the expression of the *mda-7* gene is under the control of the regulatory element; (b) introducing the linked *mda-7* gene into the tumorigenic and metastatic cells; and (c) inducing the expression of the *mda-7* gene, thereby inducing terminal differentiation in tumorigenic and metastatic cells. In an embodiment, the cell is a melanoma cell. In another embodiment, the cell is a leukemia cell. In a further embodiment, the cell is a lymphoma cell. In a still further embodiment, the cell is a neuroblastoma cell. In another still further embodiment, the cell is a glioblastoma multiforme cell.

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In a separate embodiment, the regulatory element is a promoter. In a further embodiment, the promoter is a tissue-specific promoter. In another embodiment, the promoter is an inducible promoter.

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The linked *mda-7* gene may be introduced into the cells by naked DNA technology, retroviral vectors, antibody-coated liposomes, mechanical or electrical means. These technologies are known in the art.

10

This invention also provides a method of determining the stage of a melanoma comprising: (a) obtaining appropriate amount of cells from the melanoma; (b) measuring the expression level of the *mda-7* gene in the cells; and (b) comparing the expression level with predetermined standards of normal and melanoma cells in different stages, thereby determining the stage of a melanoma.

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In an embodiment, the expression is measured by the antibodies against the *mda-7* protein. In another embodiment, the expression is measured by in situ hybridization.

20

This invention also provides a method for indicating the effectiveness of a treatment against cancer comprising measuring the expression level of *mda-7* gene in the cells of the cancer, the increase of the expression level indicating the effectiveness of the treatment. The cancer may be a melanoma, leukemia, lymphoma, neuroblastoma or a glioblastoma multiforme tumor.

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This invention provides a method of determining whether a cell is senescent comprising detecting the expression of *mda-7*, the expression of the *mda-7* gene indicating that the cell is senescent.

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5 This invention provides a method of identifying a compound inhibiting senescence comprising: a) incubating a plurality of cells with an appropriate amount of a compound; b) detecting the expression of *mda-7*, the inhibition of the expression of *mda-7* indicating that the compound is inhibiting senescence.

10 This invention provides an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-8*. In an embodiment, the nucleic acid is a cDNA. In another embodiment, the nucleic acid is genomic DNA.

15 In a separate embodiment, the *mda-8* gene is coding for a human protein.

20 This invention provides a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid molecule of an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-8*.

25 This invention provides a method of detecting the expression of *mda-8* gene in a cell comprising: a) isolating the nucleic acids in the cell; b) hybridizing the isolated nucleic acids with the nucleic acid molecules capable of *mda-8*, under conditions permitting hybrids formation; and c) detecting hybrids formed, the
30 detection of the hybrids formed indicating the expression of *mda-8* gene in the cell.

35 This invention provides a method for distinguishing a glial cell from a malignant astrocytoma cell comprising detecting the expression of *mda-8* gene using the above-

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described method, the expression of *mda-8* gene indicating that the cell is a normal glial cell.

5 The invention also provides a method for monitoring the response of a cell to an anticancer agent such as actinomycin D, adriamycin or cis-platinum comprising detecting the expression of *mda-8* gene using the above-described method, the expression of *mda-8* gene indicating that the cell responds to the anticancer agent.

10 The invention further provides a method for monitoring the response of a cell to DNA damage induced by UV irradiation comprising detecting the expression of *mda-8* gene using the above-described method, the expression of
15 *mda-8* gene indicating that the cell responds to the DNA damage.

This invention provides a method for detecting type II interferons in a sample comprising: a) incubating the
20 sample with a target cell containing the 5' regulatory element of *mda-8* permitting binding of type II interferon transcriptional regulatory proteins to the 5' regulatory element; and b) detecting the binding, the binding indicating the presence of type II interferons in the
25 sample.

In an embodiment, the cell is an eukaryotic cell.

In another embodiment, the 5' regulatory element is
30 linked to the native *mda-8* gene and the detection of binding is by the examination of the elevated level of *mda-8* gene expression.

In one embodiment, the 5' regulatory element is linked to
35 a marker gene. In a still further embodiment, the marker

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gene is β -galactosidase, luciferase or CAT.

5 This invention provides a method for identifying a compound capable of inducing terminal differentiation in human melanoma cells comprising: a) inducing appropriate concentration of the human melanoma cells with an appropriate concentration of the compound; and b) detecting the expression of *mda-8* using the above-described method, the expression of *mda-8* gene indicating
10 that the compound is capable of inducing terminal differentiation in human melanoma cells.

15 This invention provides an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-9*. In an embodiment, the nucleic acid is a cDNA. In another embodiment, the nucleic acid is genomic DNA.

20 In a separate embodiment, the *mda-9* gene is coding for a human protein.

25 This invention provides an isolated nucleic acid molecule of an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-9*, wherein the protein is a human protein.

30 This invention provides a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid molecule of an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-9*.

35 This invention provides a method of detecting the expression of *mda-9* gene in a cell comprising: a)

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isolating the nucleic acids in the cell; b) hybridizing the isolated nucleic acids with the nucleic acid molecules of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid molecule of an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-9* under conditions permitting hybrids formation; and c) detecting hybrids formed, detection of hybrids formed indicating the expression of *mda-9* gene in the cell.

This invention provides a method for indicating the stage of progression of a human melanoma cell comprising detecting the expression of *mda-9* gene using the method of detecting the expression of *mda-9* gene in a cell comprising: a) isolating the nucleic acids in the cell; b) hybridizing the isolated nucleic acids with the nucleic acid molecules of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid molecule of an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-9* under conditions permitting hybrids formation; and c) detecting hybrids formed, detection of hybrids formed indicating the expression of *mda-9* gene in the cell, the expression of *mda-9* gene indicating the stage of progression of the human melanoma cell.

This invention provides a method for identifying a compound capable of inducing terminal differentiation in human melanoma cells comprising: a) incubating appropriate concentration of the human melanoma cells with an appropriate concentration of the compound; b) detecting the expression of *mda-9* using the method of detecting the expression of *mda-9* gene in a cell

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comprising: a) isolating the nucleic acids in the cell;
b) hybridizing the isolated nucleic acids with the
nucleic acid molecules of at least 15 nucleotides capable
of specifically hybridizing with a sequence of the
5 nucleic acid molecule of an isolated nucleic acid
molecule encoding a protein produced by a melanoma
differentiation associated gene designated *mda-9* under
conditions permitting hybrids formation; and c) detecting
hybrids formed, detection of hybrids formed indicating
10 the expression of *mda-9* gene in the cell, the expression
of *mda-9* gene indicating that the compound is capable of
inducing terminal differentiation in human melanoma
cells.

15 This invention provides a method for identifying a
compound capable of inducing specific patterns of DNA
damage caused by UV irradiation and gamma irradiation in
human melanoma cells comprising: a) inducing appropriate
concentration of the human melanoma cells with an
20 appropriate concentration of the compound; and b)
detecting the expression of *mda-9* using the method of
detecting the expression of *mda-9* gene in a cell
comprising: a) isolating the nucleic acids in the cell;
b) hybridizing the isolated nucleic acids with the
25 nucleic acid molecules of at least 15 nucleotides capable
of specifically hybridizing with a sequence of the
nucleic acid molecule of an isolated nucleic acid
molecule encoding a protein produced by a melanoma
differentiation associated gene designated *mda-9* under
30 conditions permitting hybrids formation; and c) detecting
hybrids formed, detection of hybrids formed indicating
the expression of *mda-9* gene in the cell, the expression
of *mda-9* gene indicating that the compound is capable of
inducing specific patterns of DNA damage caused by UV
35 irradiation and gamma irradiation in human melanoma cells.

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5 The invention also provides a method for identifying the presence of tumor necrosis factor or a similarly acting agent comprising detecting the expression of *mda-9* gene using the above-described method, the expression of *mda-9* gene indicating that the tumor necrosis factor or similar agent is present.

10 The invention further provides a method for monitoring the response of a cell to an anticancer agent such as phenyl butyrate or VP-16 comprising detecting the expression of *mda-9* gene using the above-described method, the expression of *mda-9* gene indicating that the cell responds to the anticancer agent.

15 In a separate embodiment, the *mda-9* gene is coding for a human protein.

20 This invention provides a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid molecule of *mda-9*.

25 This invention provides a method of detecting the expression of *mda-9* gene in a cell comprising: a) isolating the nucleic acids in the cell; b) hybridizing the isolated nucleic acid with the nucleic acid capable of specifically hybridizing with *mda-9* under conditions permitting hybrid formation and c) detecting hybrids formed, detection of hybrids formed indicating the expression of *mda-9* gene in the cell.

30 This invention provides a method for distinguishing an early stage and more progressed human melanoma cell comprising detecting the expression of *mda-9* gene indicating that the cell is more progressed human
35 melanoma cell.

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This invention provides a method for identifying a compound capable of inducing terminal differentiation in human melanoma cells comprising: a) incubating human melanoma cell with the compound effective of inducing terminal differentiation in human melanoma cells; and b) detecting the expression of *mda-9* using the above-described method, the expression of *mda-9* gene indicating that the compound is capable of inducing terminal differentiation in human melanoma cells.

10

This invention provides a method for identifying a compound capable of inducing specific patterns of DNA damage caused by UV irradiation and gamma irradiation in human melanoma cells comprising: a) incubating human melanoma cells with the compound effective of inducing specific patterns of DNA damage caused by UV irradiation and gamma irradiation in human melanoma cells; and b) detecting the expression of *mda-9* using the above-described method, the expression of *mda-9* gene indicating that the compound is capable of inducing specific patterns of DNA damage caused by UV irradiation and gamma irradiation in human melanoma cells.

15
20

This invention provides an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-11*. In an embodiment, the nucleic acid is a cDNA. In another embodiment, the nucleic acid is genomic DNA.

25
30

In a separate embodiment, the *mda-11* gene is coding for a human protein.

This invention provides an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-14*. In an embodiment, the

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nucleic acid is a cDNA. In another embodiment, the nucleic acid is genomic DNA.

5 In a separate embodiment, the *mda-14* gene is coding for a human protein.

10 This invention provides an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-17*. In an embodiment, the nucleic acid is a cDNA. In another embodiment, the nucleic acid is genomic DNA.

15 In a separate embodiment, the *mda-17* gene is coding for a human protein.

20 This invention provides an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-18*. In an embodiment, the nucleic acid is a cDNA. In another embodiment, the nucleic acid is genomic DNA.

In a separate embodiment, the *mda-18* gene is coding for a human protein.

25 In an embodiment, the above-described isolated nucleic acid molecule is cDNA. In another embodiment, the nucleic acid molecule is from humans.

30 This invention also encompasses DNAs and cDNAs which encode amino acid sequences which differ from those of protein encoded by the melanoma differentiation associated genes, but which should not produce phenotypic changes. Alternatively, this invention also encompasses DNAs and cDNAs which hybridize to the DNA and cDNA of the
35 subject invention. Hybridization methods are well known

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to those of skill in the art.

The DNA molecules of the subject invention also include DNA molecules coding for polypeptide analogs, fragments or derivatives of antigenic polypeptides which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (deletion analogs containing less than all of the residues specified for the protein, substitution analogs wherein one or more residues specified are replaced by other residues and addition analogs where in one or more amino acid residues is added to a terminal or medial portion of the polypeptides) and which share some or all properties of naturally-occurring forms. These molecules include: the incorporation of codons "preferred" for expression by selected non-mammalian hosts; the provision of sites for cleavage by restriction endonuclease enzymes; and the provision of additional initial, terminal or intermediate DNA sequences that facilitate construction of readily expressed vectors.

The DNA molecules described and claimed herein are useful for the information which they provide concerning the amino acid sequence of the polypeptide and as products for the large scale synthesis of the polypeptide by a variety of recombinant techniques. The molecule is useful for generating new cloning and expression vectors, transforming and transfecting prokaryotic and eukaryotic host cells, and new and useful methods for cultured growth of such host cells capable of expression of the polypeptide and related products.

Moreover, the isolated nucleic acid molecules encoding a protein coded by the melanoma differentiation associated gene are useful for the development of probes to study

melanoma differentiation. In addition, the isolated nucleic acid molecules encoding a protein coded by the melanoma differentiation associated gene are useful for screening for agents with anticancer activity, agents which can induce DNA damage and agents which induce cell growth arrest in human melanoma.

The isolated nucleic acid molecules encoding a protein coded by the melanoma differentiation associated gene are also useful for distinguishing normal from malignant central nervous system cells, adenocarcinomas from carcinomas, and fibroblasts from epithelial cells.

This invention further provides a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of the above-described nucleic acid molecule, i.e., the melanoma differentiation associated genes.

This nucleic acid molecule produced can either be DNA or RNA. As used herein, the phrase "specifically hybridizing" means the ability of a nucleic acid molecule to recognize a nucleic acid sequence complementary to its own and to form double-helical segments through hydrogen bonding between complementary base pairs.

When a situation arises that requires the nucleic acid molecule to be uniquely recognizing a gene, it is well-known in the art to select regions in the sequence which will distinguish one gene from the other. Simple experiments may be designed to find such unique regions.

This nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of the above-described nucleic acid molecule can be used as

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a probe. Nucleic acid probe technology is well known to those skilled in the art who will readily appreciate that such probes may vary greatly in length and may be labeled with a detectable label, such as a radioisotope or fluorescent dye, to facilitate detection of the probe. DNA probe molecules may be produced by insertion of a DNA molecule which encodes a protein produced by a melanoma differentiation associated gene into suitable vectors, such as plasmids or bacteriophages, followed by transforming into suitable bacterial host cells, replication in the transformed bacterial host cells and harvesting of the DNA probes, using methods well known in the art. Alternatively, probes may be generated chemically from DNA synthesizers.

RNA probes may be generated by inserting the above-described isolated nucleic acid molecule downstream of a bacteriophage promoter such as T3, T7 or SP6. Large amounts of RNA probe may be produced by incubating the labeled nucleotides with the linearized fragment containing the above-described molecule where it contains an upstream promoter in the presence of the appropriate RNA polymerase.

This invention also provides a method of detecting expression of a melanoma differentiation associated gene in a cell which comprises obtaining total cellular RNA or mRNA from the cell, contacting the total cellular RNA or mRNA so obtained with a labelled nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of the above-described nucleic acid molecule under hybridizing conditions, determining the presence of total cellular RNA or mRNA hybridized to the molecule, and thereby detecting the expression of the melanoma differentiation associated

gene in the cell.

The nucleic acid molecules synthesized above may be used to detect expression of melanoma differentiation associated genes by detecting the presence of the correspondent RNA or mRNA. Total cellular RNA or mRNA from the cell may be isolated by many procedures well known to a person of ordinary skill in the art. The hybridizing conditions of the labelled nucleic acid molecules may be determined by routine experimentation well known in the art. The presence of total cellular RNA or mRNA hybridized to the probe may be determined by gel electrophoresis or other methods known in the art. By measuring the amount of the hybrid made, the expression of the melanoma differentiation associated genes by the cell can be determined. The labelling may be radioactive. For an example, one or more radioactive nucleotides can be incorporated in the nucleic acid when it is made.

In one embodiment of this invention, nucleic acids are extracted from lysed cells and the mRNA is isolated from the extract using an oligo-dT column which binds the poly-A tails of the mRNA molecules. The mRNA is then exposed to radioactively labelled probe on a nitrocellulose membrane, and the probe hybridizes to and thereby labels complementary mRNA sequences. Binding may be detected by luminescence autoradiography or scintillation counting. However, other methods for performing these steps are well known to those skilled in the art, and the discussion above is merely an example.

This invention also provides a method of detecting expression of a melanoma differentiation associated gene in tissue sections which comprises contacting the tissue sections with a labelled nucleic acid molecule of at

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least 15 nucleotides capable of specifically hybridizing with a sequence of the above-described nucleic acid molecule under hybridizing conditions, determining the presence of mRNA hybridized to the molecule, and thereby
5 detecting the expression of the melanoma differentiation associated gene in tissue sections.

This invention also provides the above-describe nucleic acid molecules operatively linked to a promoter of RNA
10 transcription.

This invention provides vectors which comprise the above-described isolated nucleic acid molecules. In an embodiment, the vector is a plasmid.

15 Various vectors including plasmid vectors, cosmid vectors, bacteriophage vectors and other viruses are well known to ordinary skilled practitioners. This invention further provides a vector which comprises the isolated
20 nucleic acid molecule encoding a protein produced by a melanoma differentiation gene.

As an example to obtain these vectors, insert and vector DNA can both be exposed to a restriction enzyme to create
25 complementary ends on both molecules which base pair with each other and are then ligated together with DNA ligase. Alternatively, linkers can be ligated to the insert DNA which correspond to a restriction site in the vector DNA, which is then digested with the restriction enzyme which
30 cuts at that site. Other means are also available and known to an ordinary skilled practitioner.

In an embodiment, the nucleic acid molecule is cloned in the XhoI/EcoRI site of pBlueScript*. Plasmids, mda-1,
35 mda-4, mda-5, mda-6, mda-7, mda-8, mda-9, mda-11, mda-14,

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mda-17, and mda-18 were deposited on October 26, 1993 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. Plasmids, mda-1, mda-4, mda-5, mda-6, mda-7, mda-8, mda-9, mda-11, mda-14, mda-17, and mda-18 were accorded ATCC Accession Numbers 75582, 75583, 75584, 75585, 75586, 75587, 75588, 75589, 75590, 75591 and 75592 respectively.

In another embodiment, a 3' fragment of the mda-6 gene is cloned in the EcoRI and XbaI site of the pBluescript plasmid and designed as mda-6.3'. The mda-6.3' was deposited on September 30, 1994 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. Plasmid, mda-6.3' was accorded ATCC Accession Numbers 75903.

In another embodiment, a 5' fragment of the mda-6 gene is cloned in the salI site of the pSP64 plasmid and designed as mda-6.5'. The mda-6.5' was deposited on September 30, 1994 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. Plasmid, mda-6.5' was accorded ATCC Accession Number 75904.

Plasmids mda-6.3' and mda-6.5' constitute the full-length of the mda-6 gene. An ordinary skilled artisan can

easily obtain the inserts from the plasmids and ligate the inserts to obtain the full-length gene.

5 In another embodiment, a 3' fragment of the *mda-7* gene is cloned in the EcoRI and XbaI site of the pBluescript plasmid and designed as *mda-7.3'*. The *mda-7.3'* was deposited on September 30, 1994 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of
10 the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. Plasmid, *mda-7.3'* was accorded ATCC Accession Number 75905.

15 In another embodiment, a 5' fragment of the *mda-7* gene is cloned in the *salI* site of the pSP64 plasmid and designed as *mda-7.5'*. The *mda-7.5'* was deposited on September 30, 1994 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A.
20 under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. Plasmid, *mda-7.5'* was accorded ATCC Accession Numbers 75906.

25 Plasmids *mda-7.3'* and *mda-7.5'* constitute the full-length of the *mda-7* gene. An ordinary skilled artisan can easily obtain the inserts from the plasmids and ligate the inserts to obtain the full-length gene.

30 This invention provides a host vector system for the production of a polypeptide having the biological activity of a protein encoded by melanoma differentiation associated gene which comprises the above-described vector and a suitable host. These vectors may be
35 transformed into a suitable host cell to form a host cell

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vector system for the production of a protein produced by the melanoma differentiation associated genes.

5 Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector includes a promoter such as the lac promoter and for transcription initiation the Shine-Dalgarno sequence and the start
10 codon AUG. Similarly, a eukaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors may be obtained
15 commercially or assembled from the sequences described by methods well known in the art, for example the methods described above for constructing vectors in general. Expression vectors are useful to produce cells that
20 express the protein produced by the melanoma differentiation associated genes.

This invention further provides an isolated DNA or cDNA molecule described hereinabove wherein the host cell is selected from the group consisting of bacterial cells
25 (such as E.coli), yeast cells, fungal cells, insect cells and animal cells. Suitable animal cells include, but are not limited to Vero cells, HeLa cells, Cos cells, CV1 cells and various primary mammalian cells.

30 As stated above and in the text which follows, this invention provides gene(s) which express when a cell becomes terminally differentiated and irreversibly growth arrested, treated with a DNA damaging agent and/or exposed to an anticancer agent. Therefore, this
35 invention is useful for inducing a target cell to a

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terminally differentiated stage. Such a target cell may be a cancerous cell such as a melanoma cell or a glioblastoma multiforme cell. The gene which expresses when a cell becomes terminally differentiated may be introduced into the target cell via retroviral technology or other technologies known in the art. The gene may be controlled by its own promoter or other heterologous promoters. Expression of this gene will then result in terminal differentiation and an irreversible loss of proliferative capacity in the cancerous cell.

This invention has also provided nucleic acid molecules which will suppress the terminal differentiation of a cell. Such molecules are useful for preventing terminal differentiation by switching off a target gene in a cell which has been treated with differentiation inducing agents, anticancer agents or DNA damaging agents. The target gene may be switched off via antisense technology. After the gene has been switched off, normal cells, such as bone marrow stem cells, can be prevented from becoming terminally differentiated and irreversibly growth arrested when treated with differentiation inducing agents, anticancer agents or DNA damaging agents.

Antisense technology is well known in the art. Essentially, a segment of the melanoma differentiation associated gene will be selected to be the antisense sequence. The expression of the antisense sequence will switch off the expression of the gene. The antisense sequence may be introduced into the cell via technologies which are well known in the art, such as electroporation transduction, retroviral insertion or liposome-mediated gene transfer.

This invention also provides a method of producing a

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polypeptide having the biological activity of a protein encoded by melanoma differentiation associated gene which comprises growing the host cells of the above-described host vector system under suitable conditions permitting production of the polypeptide and recovering the polypeptide so produced.

This invention provides a mammalian cell comprising a DNA molecule encoding a protein produced by a melanoma differentiation associated gene, such as a mammalian cell comprising a plasmid adapted for expression in a mammalian cell, which comprises a DNA molecule encoding a protein produced by the melanoma differentiation associated gene and the regulatory elements necessary for expression of the DNA in the mammalian cell so located relative to the DNA encoding a protein produced by a melanoma differentiation gene as to permit expression thereof.

Numerous mammalian cells may be used as hosts, including, but not limited to, the mouse fibroblast cell NIH3T3, CREF cells, CHO cells, HeLa cells, Ltk⁻ cells, Cos cells, etc. Expression plasmids such as that described supra may be used to transfect mammalian cells by methods well known in the art such as calcium phosphate precipitation, electroporation or the plasmid may be otherwise introduced into mammalian cells, e.g., by microinjection, to obtain mammalian cells which comprise DNA, e.g., cDNA or a plasmid, encoding a protein produced by a melanoma differentiation associated gene.

Also provided by this invention is a purified protein encoded by the above-described isolated nucleic acid molecule. As used herein, the term "purified protein"

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shall mean isolated naturally-occurring protein encoded by a melanoma differentiation associated gene (purified from nature or manufactured such that the primary, secondary and tertiary conformation, and posttranslational modifications are identical to naturally-occurring material) as well as non-naturally occurring polypeptides having a primary structural conformation (i.e. continuous sequence of amino acid residues). Such polypeptides include derivatives and analogs.

This invention also provides a method to produce antibody using the above purified protein. In an embodiment, the antibody is monoclonal. In another embodiment, the antibody is polyclonal.

This invention further provides antibodies capable of binding to the purified protein produced by the melanoma differentiation associated genes.

With the protein sequence information which can either be derived from the above described nucleic molecule or by direct protein sequencing of the above described purified protein, antigenic areas may be identified and antibodies directed against these areas may be generated and targeted to the cancer for imaging the cancer or therapies.

This invention provides a method to select specific regions on the protein produced by a melanoma differentiation associated gene to generate antibodies. Amino acid sequences may be analyzed by methods well known to those skilled in the art to determine whether they produce hydrophobic or hydrophilic regions in the proteins which they build. In the case of cell membrane

proteins, hydrophobic regions are well known to form the part of the protein that is inserted into the lipid bilayer of the cell membrane, while hydrophilic regions are located on the cell surface, in an aqueous environment. Usually, the hydrophilic regions will be more immunogenic than the hydrophobic regions. Therefore the hydrophilic amino acid sequences may be selected and used to generate antibodies specific to protein produced by the melanoma differentiation genes.

10 The selected peptides may be prepared using commercially available machines. As an alternative, DNA, such as a cDNA or a fragment thereof, may be cloned and expressed and the resulting polypeptide recovered and used as an immunogen.

15 Polyclonal antibodies against these peptides may be produced by immunizing animals using the selected peptides. Monoclonal antibodies are prepared using hybridoma technology by fusing antibody producing B cells from immunized animals with myeloma cells and selecting the resulting hybridoma cell line producing the desired antibody. Alternatively, monoclonal antibodies may be produced by in vitro techniques known to a person of ordinary skill in the art. These antibodies are useful to detect the expression of protein produced by the melanoma differentiation associated genes in living animals, in humans, or in biological tissues or fluids isolated from animals or humans.

25
30 This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

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Experimental Details

First Series of Experiments

5 MATERIALS AND METHODS

Cell Line, Growth Conditions, and Preparation of Conditioned Medium

10 The H0-1 cell line is a melanotic melanoma derived from a 49-year-old female and was used between passage 100 and 125 (6,8,13). H0-1 cells were kindly provided by Dr. Beppino C. Giovanella, Stehlin Foundation for Cancer Research, Houston, Texas. Cultures were grown at 37 °C
15 in a 95% air 5% CO₂-humidified incubator in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (DMEM-10) (Hyclone*, Logan, UT). H0-1 cells were maintained in the logarithmic stage of growth by subculturing (1:5 or 1:10) prior to confluency
20 approximately every 4 to 5 days. The effect of IFN-β (2000 units/ml), IFN-γ (2000 units/ml), IFN-β + IFN-γ (1000 units/ml of each interferon), MPA (3.0 μM), RA (2.5 μM), MEZ (10 ng/ml), IFN-β + MEZ (2000 units/ml + 10 ng/ml), MPA + MEZ (3.0 μM + 10 ng/ml, and RA + MEZ (2.5
25 μM + 10 ng/ml) on growth was determined after 4 days of treatment, as described previously (6,8,9). Terminal cell differentiation, with a concomitant loss of proliferative capacity, was assayed by treating cells with the various agents for either 4 or 7 days, washing
30 cultures two times with serum-free DMEM, followed by incubation in DMEM-10 in the absence of inducer(s) for an additional 14 days. Total cell numbers were determined after days 4, 7, 14, and 21, using a Z_M Coulter Counter*, and viable cell numbers were determined by trypan blue

* Trade-mark

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dye exclusion (6). Terminal cell differentiation was indicated if no proliferation occurred, but cells remained viable, after growth for 14 days, with medium changes every 3 or 4 days, in the absence of the inducer(s). Increases in cell number (two or more population doublings) after removal of the inducing agent(s) was considered reversible growth suppression. Conditioned medium was prepared from H0-1 cells treated with a high dose of MEZ (50 ng/ml), IFN- β + MEZ (2000 U/ml + 10 ng/ml), MPA + MEZ (3.0 μ M + 10 ng/ml), or RA + MEZ (2.5 μ + 10 ng/ml) for 24 hours followed by three washes in DMEM without FBS and growth for 72 hours in inducer-free medium (DMEM-10). Conditioned medium was collected from treated cultures, contaminating cells were removed by centrifugation at 1000 rpm/10 min., and conditioned medium was stored at 4°C until assayed for gene modulatory activity. Control conditioned medium was obtained as experimental conditioned medium from cells receiving only a medium change 24 hours after plating in the absence of test compound(s) and growth for 72 hours in DMEM-10.

RNA Isolation and Northern Hybridization Analysis

Steady-state levels of specific mRNAs were determined by Northern blotting analysis of total RNA probed with appropriate ^{32} P-labeled gene probes as described previously (14-16). RNAs were analyzed from cells treated with inducer for 24 hours, treated for 24 hours with inducer followed by growth for 72 hours in the absence of inducer, or treated continuously for 96 hours with the inducer. The concentration of inducing compounds used were the same as those used for growth studies. The effect of conditioned medium on gene

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expression changes was determined by treating H0-1 cells for 24 hours with a 1:2 dilution of conditioned medium (equal volumes of conditioned medium and DMEM supplemented with 10% fetal bovine serum (DMEM-10) or for 5 96 hours with a 1:4 dilution of conditioned medium (1 vol. of conditioned medium to 3 vol. of DMEM-10). The probes used in the present study were specific for β -actin (17), γ -actin (17), c-jun (18), c-myc (19), fibronectin (20), gro/MGSA (21), HLA Class I antigen 10 (22), HLA Class II antigen (HLA-DR₈) (22), α_5 integrin (23), β_1 integrin (24), ISG-15 (25), ISG-54 (25), jun-B (26), and tenascin (27). Northern blots were also probed with a ³²P-labeled GAPDH gene (15) to verify similar mRNA expression under the various experimental conditions. 15 Following hybridization, the filters were washed and exposed for autoradiography.

Reagents

20 Recombinant human IFN- β , with a serine substituted for a cysteine at position 17 of the molecule (28), was kindly provided by Triton Bioscience (Alameda, CA). IFN- β was obtained as a lyophilized powder with a concentration of 4.5×10^7 U/ml. Recombinant human immune interferon (IFN- 25 γ) was produced and purified to homogeneity as described previously (29). IFN- γ was kindly provided by Dr. Sidney Pestka, UMDNJ-Robert Wood Johnson Medical School, Piscataway, New Jersey. The interferon titers were determined using a cytopathic effect inhibition assay 30 with vesicular stomatitis virus on a bovine kidney cell line (MDBK) or human fibroblast AG-1732 cells (30). Concentrated stocks of IFN- β and IFN- γ were diluted to 1×10^6 U/ml in DMEM-10, frozen at -80°C, thawed immediately prior to use, and diluted to the appropriate

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concentration in DMEM-10. MEZ, RA, and MPA were obtained from Sigma Scientific Co. (St. Louis, MO). Stock solutions were prepared in dimethyl sulfoxide (DMSO), aliquoted into small portions, and stored at -20°C. The
5 final concentration of DMSO used in solvent controls was 0.01%. This concentration of DMSO did not alter growth, melanin synthesis, tyrosinase activity, or antigen expression in HO-1 cells.

10 EXPERIMENTAL RESULTS

Induction of Reversible and Irreversible Growth Suppression (Terminal Cell Differentiation) in HO-1 Cells

15 The effect of RA, MPA, MEZ, IFN- β and IFN- γ , used alone or in various combinations, on growth (reversible and irreversible growth suppression), melanin synthesis, tyrosinase activity, and cellular morphology of HO-1
20 cells is summarized in Table 1. The most effective agents in inhibiting HO-1 growth were the combinations of IFN- β + MEZ and IFN- β + IFN- γ (Figure 1). The relative order of antiproliferative activity of the remaining
25 compounds was MPA = MPA + MEZ > IFN- γ > MEZ = RA + MEZ. In the case of RA, no inhibition of growth occurred. Treatment of HO-1 cells with IFN- β + MEZ for 96 hours
30 resulted in an irreversible loss of proliferative capacity, that is, terminal cell differentiation. This was indicated by the failure of treated cells to resume growth, even though they remained viable, after removal
of the test agents. In contrast, all of the other compounds resulted in a reversible inhibition of growth (data not shown). These findings indicate a dissociation
between growth suppression and terminal cell differentiation in HO-1 cells.

Treatment of H0-1 cells with MPA, MEZ, RA + MEZ, MPA + MEZ, or IFN- β + MEZ resulted in distinctive morphologic changes that were characterized by dendrite-like processes (data not shown) (6,8,10). RA, MPA, MEZ, IFN- β , and IFN- β + MEZ have been shown previously to enhance melanin synthesis, a marker of melanoma differentiation, in H0-1 cells (6,8,12). In contrast, IFN- γ , alone or in combination with IFN- β , did not induce morphologic changes or increase melanin levels above

TABLE 1

Experimental Conditions ^a	Morphology changes ^b	Melanin Synthesis ^c	Tyrosinase activity ^d
RA (2.5 μ M)	-	1+	2+
MPA (3.0 μ M)	+	2+	3+
MEZ (10 ng/ml)	+	1+	NT
IFN- β (2000 U/ml)	-	1+	NT
IFN- γ (2000 U/ml)	-	-	NT
RA + MEZ (2.5 μ M + 10 ng/ml)	+	NT	NT
MPA + MEZ (3.0 μ M + 10 ng/ml)	+	NT	NT
IFN- β + IFN- γ (1000 U/ml + 1000 U/ml)	-	1+	NT
IFN- β + MEZ (2000 U/ml + 10 ng/ml)	+	4+	NT

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TABLE 1 (Continued)

	Experimental conditions ^a	Growth Suppression (reversible) ^c	Terminal cell differentiation ^f
5	RA (2.5 μ M)	-	-
	MPA (3.0 μ M)	3+	-
10	MEZ (10 ng/ml)	1+	-
	IFN- β (2000 U/ml)	3+	-
15	IFN- γ (2000 U/ml)	2+	-
20	RA + MEZ (2.5 μ M + 10 ng/ml)	1+	-
	MPA + MEZ (3.0 μ M + 10 ng/ml)	3+	-
25	IFN- β + IFN- γ (1000 U/ml + 1000 U/ml)	4+	-
30	IFN- β + MEZ (2000 U/ml + 10 ng/ml)	4+ ^g	+

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^a H0-1 cells were grown for 96 hr or for 6 or 7 days (with medium changes after 3 or 4 days) in the presence of the agents indicated. For morphology, cells grown for 96 hr in the test agent were observed microscopically. For melanin synthesis, results are for 6-d assays for RA and MPA (11) or 7-d assays for MEZ, IFN- β , IFN- γ , IFN- β + IFN- γ and IFN- β + MEZ (6,12). For tyrosinase assays, results are for 6-d assays for RA, MPA and MEZ (10). Growth suppression (reversible and terminal cell differentiation) assays, refer to cultures treated with the indicated compound(s) for 96 hr prior to cell number determination, or treated for 96 hr and then grown for 2 weeks (with medium changes every 4 days) in the absence of compound prior to cell

number determination.

5 ^b Morphology changes refer to the development of dendrite-like processes 96 hr after growth in the indicated compound. + = presence of dendrite-like processes; - = no dendrite-like processes.

10 ^c Melanin assays were determined as described in refs. 6,8,11,12. Results are expressed as relative increases based on separate data presented in refs. 6,8,11,12. N.T. = not tested.

15 ^d Tyrosinase assays were performed as described in ref. 11. Relative increases (of a similar magnitude) were found for RA, MPA and MEZ after 6 days exposure to these agents (11). N.T. = not tested.

20 ^e Reversible growth suppression indicates resumption of cell growth after treatment with the indicated compound(s) for 96 hr, removal of the test agent and growth for 14 days in compound(s) free medium. Further details can be found in materials and methods. The degree of initial 96 hr growth suppression is indicated as: - = no significant change in growth (< 10% reduction in growth in comparison with untreated control cultures); 1+ = ~30% reduction in growth in comparison with untreated control cultures; 2+ = ~40% reduction in growth in comparison with untreated control cultures; 3+ = ~50 to 60% reduction in growth in comparison with untreated control cultures; 4+ = ~80% reduction in growth in comparison with untreated control cultures.

35 ^f The combination of IFN- β + MEZ results in irreversible growth suppression.

40 ^g The combination of IFN- β + MEZ results in irreversible growth suppression

45 that induced by IFN- β alone (10,12). With the exception of IFN- β + MEZ, morphologic and melanin changes were reversible following removal of the test agent(s) (data not shown). These data indicate that specific cellular and biochemical changes induced in H0-1 cells, such as reversible growth suppression, melanin synthesis, and morphologic changes, can occur with or without the

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induction of terminal cell differentiation. However, an irreversible loss of proliferative capacity with the retention of cell viability is a property unique to the terminal cell differentiation phenotype.

5

Changes in the Expression of Early Growth Response and Interferon-Responsive Genes During Reversible and Irreversible Growth Suppression (Terminal Cell Differentiation) in H0-1 Cells

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Initial studies were conducted to determine the effect of the various differentiation and growth modulating agents on the 96-hour expression of the early response genes *c-fos*, *c-jun*, *jun-B*, *jun-D*, and *c-myc* (Figure 2). None of the experimental treatments resulted in altered *c-fos* expression and no hybridization was obtained with RNA isolated from control or treated cells probed with *jun-D* (data not shown). Increases were observed, however, in both *c-jun* and *jun-B* expression in H0-1 cells treated for 96 hours with all of the test agents, with the exception of IFN- β and RA (see Figure 2). The magnitude of the increase was similar in H0-1 cells treated with IFN- γ , MEZ, or MPA and was greatest for H0-1 cells treated with IFN- β + MEZ, MPA + MEZ, or RA + MEZ (see Figure 2). Unlike *c-jun* and *jun-B* expression *c-myc* expression was downregulated in H0-1 cells grown for 96 hours in MEZ, MPA, IFN- β + MEZ, MPA + MEZ, and RA + MEZ (Figure 2). The magnitude of suppression was greater in H0-1 cells treated with IFN- β + MEZ, MPA + MEZ, and RA + MEZ. In contrast, treatment of H0-1 cells with IFN- γ , alone or in combination with IFN- β , resulted in increased *c-myc* expression.

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To determine the temporal relationship between treatment with IFN- β + MEZ, MPA + MEZ, and RA + MEZ and changes in

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c-jun, jun-B, and c-myc levels, HO-1 cells were treated with the inducers for 24 h and total cytoplasmic RNA was isolated and analyzed by Northern blotting (Figure 3). Because many of the effects observed when MEZ is combined with IFN- β , MPA, or RA are observed to a lesser extent in cells treated with 10 mg/ml of MEZ alone, RNA was also isolated from HO-1 cell treated for 24 h with a high dose of MEZ (50 ng/ml) (Figure 3). Under these experimental conditions, c-jun and jun-B expression were induced under all experimental conditions and c-myc expression was only marginally reduced in cultures treated with IFN- β + MEZ, MPA + MEZ, and RA + MEZ. Twenty-four-hour exposure to IFN- β + MEZ resulted in the largest induction in c-jun and jun-B expression.

To determine if the gene expression changes induced during the induction of reversible and irreversible growth suppression/differentiation persist in HO-1 cells treated with specific inducers, cultures were grown for 24 h in the presence of inducer and then incubated for an additional 72 h in inducer-free medium prior to isolating total cellular RNA (Figure 3). Under these experimental conditions, c-jun and jun-B expression were induced to the greatest extent in IFN- β + MEZ-treated cultures. Smaller increases in c-jun and jun-B expression were apparent in high-dose MEZ-, MPA + MEZ-, and RA + MEZ-treated HO-1 cells. In the case of c-myc, expression was dramatically reduced in IFN- β + MEZ-treated cultures and reduced to a lesser extent in MPA + MEZ- and RA + MEZ-treated cultures. These results suggest that the hierarchy for inducing c-jun, jun-B, and c-myc gene expression changes in HO-1 cells is IFN- β + MEZ > MPA + MEZ > RA + MEZ. As will be discussed, this same pattern of potency in inducing gene expression changes in HO-1 cells is also observed with a number of additional genes.

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5 Treatment of HO-1 cells for 96 h with the combination of IFN- β + MEZ, MPA + MEZ, or RA + MEZ resulted in the enhanced expression of the cytokine-responsive genes HLA Class I antigen and gro/MGSA (Figure 2). In contrast, treatment with IFN- β , MEZ, MPA, or RA alone did not significantly alter HLA Class I antigen gene expression or induce gro/MGSA gene expression. Treatment of HO-1 cells for 96 h with IFN- γ , alone or in combination with IFN- β , also enhanced HLA Class I antigen expression in HO-1 cells, whereas it did not induce gro/MGSA expression (Figure 2). In contrast, although a 96-h exposure of HO-1 cells to IFN- γ , alone and in combination with IFN- β , enhanced expression of the HLA Class II antigen gene (HLA-DR β), expression of this gene was not significantly enhanced by treatment with IFN- β + MEZ, MPA + MEZ, or RA + MEZ (Figure 2). These observations indicate possible autocrine loops involving both a type I interferon (leukocyte interferon IFN- α) and IFN- β as opposed to a type II interferon (IFN- γ) and gro/MGSA in the induction of gene expression changes occurring during both reversible (MPA + MEZ and RA + MEZ) and terminal cell differentiation (IFN- β + MEZ).

25 To determine if an interferon or an interferon-like molecule might be associated with the induction of reversible or irreversible differentiation or both in HO-1 cells, the effect of the various differentiation-inducing and growth-suppressing agents on expression of the interferon-responsive genes, ISG-15 and ISG-54 (25, 31, 32) were determined. As can be seen in Figure 2, treatment of HO-1 cells for 96 h with the combination of IFN- β + MEZ, MPA + MEZ, or RA + MEZ resulted in the induction in ISG-15 and ISG-54 gene expression.

Further support for a type I interferon and a gro/MGSA autocrine loop in the reversible commitment to differentiation (MPA + MEZ) and terminal cell differentiation (IFN- β + MEZ) processes in HO-1 cells was indicated by analysis of gene expression changes occurring in cultures treated with inducers for 24 h or in cultures treated with inducers for 24 h followed by growth in the absence of inducers for 72 h (Figure 3). Growth of HO-1 cells for 24 h in the presence of IFN- β + MEZ resulted in the induction or enhanced expression of the gro/MGSA, HLA Class I antigen, and ISG-15 gene. Similarly, a 24-h treatment with MPA + MEZ induced expression of gro/MGSA to a similar extent as IFN- β + MEZ, whereas the effects on HLA Class I antigen and ISG-15 expression were more modest. In contrast, a 24-h treatment with RA + MEZ or a high dose of MEZ did not induce gro/ MGSA or ISG-15 expression, but these treatments did induce a modest increase in HLA Class I antigen expression (Figure 3). Treatment of HO-1 cells for 24 h with inducer (IFN- β + MEZ, MPA + MEZ, RA + MEZ or a high dose of MEZ) followed by growth for 72 hours in the absence of inducer resulted in the following changes in HO-1 gene expression: (1) gro/MGSA was induced only by IFN- β + MEZ treatment; (2) enhanced HLA Class I antigen expression was induced by all of the treatments with the following potencies, IFN- β + MEZ > MPA + MEZ $\geq$ high dose MEZ > RA + MEZ; and (3) ISG-15 was induced to a similar extent by IFN- β + MEZ and MPA + MEZ, whereas RA + MEZ and high-dose MEZ did not induce ISG-15 expression.

Changes in the Expression of Extracellular and Extracellular Matrix Receptor Genes During Reversible and Irreversible Growth Suppression (Terminal Cell Differentiation) in HO-1 cell.

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Terminal differentiation in HO-1 cells induced by IFN- β + MEZ is associated with morphologic changes resulting in the formation of dendrite-like processes and specific biochemical changes, that is, enhanced tyrosinase activity and melanin synthesis (6). Similar morphologic and biochemical changes are induced in HO-1 cells by MEZ (6, 8) and MPA (11). Studies were conducted to determine the possible relationship between these morphologic and biochemical changes and the expression of genes encoding extracellular matrix molecules (fibronectin and tenascin), receptors for extracellular matrix proteins (α -integrin and β_1 integrin), and cytoskeleton proteins (β -actin and γ -actin). When treated for 96 h, fibronectin expression was increased under all treatment protocols, with IFN- β and RA being the least effective agents in inducing enhanced fibronectin mRNA in HO-1 cells (i.e. signals were only detected after long exposures of Northern blots) (Figure 4 and data not shown). The most effective single agents resulting in enhanced fibronectin expression were IFN- γ and MPA, whereas MEZ was less potent in inducing enhanced fibronectin expression (Figure 4). Large increases in fibronectin expression were also observed in HO-1 cells grown for 96 h in the combination of IFN- β + IFN- γ , IFN- β + MEZ, MPA + MEZ, and RA + MEZ. IFN- β + MEZ was more effective than MPA + MEZ, RA + MEZ, or a high dose of MEZ in inducing enhanced fibronectin expression after 24-h treatment (Figure 5). IFN- β + MEZ also enhanced fibronectin expression to a greater extent than MPA + MEZ, RA + MEZ, and a high dose of MEZ after removing this combination of inducers and growth for 72 h in medium devoid of the inducing agents (Figure 5).

Expression of the tenascin gene in HO-1 cells treated

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with the various differentiation-inducing and growth-suppressing agents was more complex than fibronectin (Figures 4 and 5). Growth of HO-1 cells for 96 h in the presence of IFN- β , MEZ, RA, IFN- β + MEZ, or RA + MEZ resulted in decreased tenascin expression whereas IFN- γ , MPA, and IFN- β + IFN- α resulted in enhanced tenascin expression (Figure 4). Ninety-six-hour treatment with IFN- γ , alone or in combination with IFN- β , resulted in the greatest increase in tenascin expression. In contrast, after a 24-h treatment, tenascin expression was not significantly altered by a high dose of MEZ or RA + MEZ, whereas a small increase in tenascin expression was found in IFN- β + MEZ- and MPA + MEZ-treated cultures (Figure 5). When cultures were treated for 24 h with inducer and then grown for 72 h in the absence of inducer, decreased expression of tenascin was observed in high-dose MEZ- and RA + MEZ-treated cultures, whereas only small reductions in tenascin expression were apparent in MPA + MEZ- or IFN- β + MEZ-treated HO-1 cells (Figure 5).

Changes were also observed in the expression of matrix receptor genes for extracellular matrix proteins, α_5 -integrin, β_1 -integrin in HO-1 cells grown for (1) 24 h in the presence of the inducer(s), (2) 24 h in inducer(s) followed by 72 h in the absence of inducer(s), or (3) continuously in inducer(s) for 96 h (Figures 4 and 5). Increases were observed in α_5 integrin expression in HO-1 cells treated for 96 h with IFN- β + IFN- γ , IFN- β + MEZ, MPA + MEZ and, to a lesser extent, with RA + MEZ (Figure 4). Increased α_5 integrin expression was also apparent in HO-1 cells treated continuously for 24 hrs or treated for 24 hrs followed by 72 hr growth in the absence of inducer(s) to a high dose of MEZ, MPA + MEZ, RA + MEZ, or

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IFN- β + MEZ. In contrast, α_5 integrin expressed was reduced in cultures treated with RA for 96 h. In the case of the β_1 integrin, upregulation after 96-h treatment was apparent in cells treated with IFN- γ , IFN- β + MEZ, MPA + MEZ, and RA + MEZ (Figure 4). The most effective inducer of both α_5 and β_1 integrin expression in HO-1 cells was IFN- β + MEZ (Figures 4 and 5). The level of upregulation was greater for α_5 integrin than the β_1 integrin (Figures 4 and 5).

The effect of the various growth-suppressing and differentiation-modulating compounds on expression of cytoskeletal genes (β -actin and γ -actin) in HO-1 cells is shown in Figures 4 and 5. Under most experimental conditions, only small changes were observed in β -actin and γ -actin mRNA levels. In the case of 96-h-treated cultures, both β -actin and, to a greater extent, γ -actin expression were decreased by treatment with a number of agents, resulting in growth suppression. In contrast, RA, which is not growth suppressive in HO-1 cells, did not significantly alter the expression of these cytoskeletal genes. A common change that was generally most pronounced under all three experimental protocols in HO-1 cells, that is, 24-h treatment, 24-h treatment followed by 72-h growth in the absence of inducer, or continuous treatment for 96 h, was the reduction in β -actin and γ -actin expression by IFN- β + MEZ.

Modulation of Gene Expression in HO-1 Cells by Conditioned Medium Obtained from Differentiation-Inducer-Treated HO-1 Cells.

The studies described previously demonstrated that interferon-responsive genes and the gro/MGSA gene were

activated during the process of reversible and irreversible differentiation in HO-1 cells. They further suggested the possibility of an involvement of autocrine-feedback pathways in the differentiation process (Figures 2 and 3). To determine directly if HO-1 cells treated with agents inducing a reversible commitment to differentiation (a high dose of MEZ, RA + MEZ, and MPA + MEZ) and/or terminal cell differentiation (IFN- β + MEZ) secrete factor(s) that can modulate gene expression in HO-1 cells, conditioned medium was collected from cells treated for 24 h with the inducer followed by growth for 72 h in the absence of inducer (Figures 6 and 7). HO-1 cells were grown in an equal volume of conditioned medium plus and equal volume of DMEM-10 (1:2) for 24 h or with 1 vol of conditioned medium plus 3 vol of DMEM-10 (1:4) for 96 h. Total cytoplasmic RNA was then isolated and analyzed by Northern blotting for the expression of a series of early growth response, interferon responsive, extracellular matrix, extracellular matrix receptor, and cytoskeletal genes (Figures 6 and 7). With the exception of fibronectin and small increases in β_1 integrin expression, treatment for 24 h with 1:2 conditioned medium obtained from the other experimental conditions (which result in a reversible commitment to differentiation) did not alter or induce expression of the genes tested, including c-jun, jun-B, c-myc, gro/MGSA, HLA Class I antigen, ISG-15, α_5 integrin, β -actin, γ -actin, or tenascin. Exposure of HO-1 cells for 96 h to 1:4 conditioned medium obtained from IFN- β + MEZ-treated HO-1 cells also enhanced fibronectin, HLA Class I antigen, β_1 integrin and tenascin expression, as well as inducing ISG-15 expression. With the exception of fibronectin and tenascin, which were enhanced by 1:4 conditioned medium obtained from 24-h treated IFN- β +

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MEZ, MPA + MEZ, RA + MEZ treated cultures, no modification in the expression of the various genes was apparent using 1:4 conditioned medium from any of the experimental procedures.

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The HO-1 human melanoma cell line can be chemically induced to reversibly express specific markers of differentiation or to undergo terminal cell differentiation. The present study was undertaken to determine which specific programs of gene expression are modified as a consequence of these cellular alterations. Inductions of terminal differentiation, and, to a lesser extent, reversible differentiation, was associated with changes in the expression specific immediate early response, interferon-responsive, cytokine-responsive, extracellular matrix, and extracellular matrix receptor genes. In addition, conditioned medium obtained from HO-1 cells treated with IFN- β + MEZ also resulted in similar changes in gene expression in naive HO-1 cells as those observed following direct exposure to the chemical inducers of terminal differentiation. These results indicate that common gene-expression changes are associated with both the reversible and irreversible induction of differentiation in HO-1 cells. In addition, the terminal differentiation process is correlated with the activation of several autocrine pathways involving both IFN- β and gro/MGSA.

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Immediate early response genes, such as *c-myc*, *c-fos*, *c-jun*, *jun-B*, and *jun-D*, have been shown to be involved in the regulation of growth and/or differentiation in other model systems [33-35]. In the case of *c-myc*, a reduction in expression of this gene is observed in many cell types either induced to terminally differentiate or under conditions resulting in a reduction in cellular growth

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without the induction of differentiation-related genes (36-40). A direct role of *c-myc* expression in regulating differentiation in a variety of model cell culture systems has also been demonstrated using *c-myc* antisense constructs or oligomers (41-45). In specific systems, the downregulation of *c-myc* expression by antisense constructs or oligomers has been shown to result in differentiation and growth suppression in the absence of inducing agents (45-48). Induction of both reversible differentiation and, to a greater extent, terminal differentiation in H0-1 cells resulted in decreased *c-myc* expression. Downregulation of *c-myc* expression was independent of growth suppression, as indicated by the enhanced expression of *c-myc* in IFN- β + IFN- γ treated cells, even though this combination of agents resulted in maximum growth suppression without the induction of any morphologic or biochemical markers of melanoma differentiation. Based on the temporal relationship and the magnitude of *c-myc* downregulation by the various inducing agents, continued suppression of *c-myc* expression may be required for the induction of terminal differentiation in H0-1 cells. Studies using antisense *c-myc* constructs should prove valuable in directly addressing the relationship between *c-myc* expression and terminal differentiation in H0-1 cells.

Two immediate early response genes, *c-fos* and *c-jun*, code for transcription factors involved in nuclear signal transduction (35, 46, 47). Expression of these genes can be induced by many external stimuli, including cytokines, growth factors, serum, phorbol esters, neurotransmitters, and viral infection (35, 46, 47). The proteins *c-fos* and *c-jun* can form a heterodimer as part of the AP-1 transcription-factor complex that binds efficiently to

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AP-1 sites (TGA^G/cTCA) in DNA (35, 46, 47). Previous studies have indicated that both the *c-jun* and *c-fos* genes are activated during monocytic differentiation induced by TPA, macrophage colony-stimulating factor (M-CSF), and okadaic acid (48-50). Elevation of AP-1 activity also has been demonstrated during the induction of differentiation of F9 embryonal carcinoma stem cells by RA (51). In contrast the transcriptional enhancing activity of *c-jun*, *jun-B* (which is induced by a number of external stimuli that also induce *c-jun*) functions as a negative regulator of several genes normally activated by *c-jun* (52, 53). In the process of monocytic differentiation induced by TPA in human cells, *jun-B* gene transcription, steady-state mRNA levels, and mRNA stability are enhanced (54). Similarly, *jun-B* expression is enhanced during the process of monocytic differentiation induced in murine cells by serum-free conditioned medium from mouse lungs (55). Induction of growth suppression and both reversible and irreversible differentiation in HO-1 cells are unaltered at later time points. Unlike TPA-induced monocytic differentiation (48, 54), induction of *jun-B* expression by IFN- β + MEZ in HO-1 cells is regulated only at the transcriptional level (56). These data indicate that enhanced expressions of *c-jun* and *jun-B* in HO-1 cells is not directly related to the induction of terminal differentiation in HO-1 cells. A sustained elevation of *c-jun* and *jun-B* expression, however, may be components of the differentiation program in HO-1 cells.

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The process of cellular differentiation is frequently associated with profound changes in cellular morphology that are related to cell-cell and cell-extracellular matrix interactions as well as the expression of cell

growth and cytoskeletal genes (57). In addition, cell shape and cell-extracellular matrix interactions also play important roles in the process of tumorigenesis and metastasis (58, 59). Transformed cells often exhibit reductions in fibronectin expression as well as decreases in expression of specific integrin genes, which encode receptors for extracellular matrix proteins (60, 61). Of particular recent interest is the $\alpha_5\beta_1$ integrin complex that appears to be the major receptor for fibronectin (62). Decreases in α_5 β_1 expression have been found in oncogenically transformed cells (60) and overexpression of the combination of α_5 and β_1 integrin cDNA in Chinese hamster ovary (CHO) cells results in a direct suppression of the transformed phenotype (61). Agents that induced reversible differentiation (MPA + MEZ and RA + MEZ), irreversible differentiation (IFN- β + MEZ), and increased growth suppression without inducing markers of differentiation (IFN- β + IFN- γ) in HO-1 cells enhanced fibronectin, α_5 integrin, and β_1 integrin expression. These findings suggest that the specific combinations of cytokines, such as IFN- β + IFN- γ , resulting in growth suppression and combinations of agents that induce either a reversible commitment to differentiation or terminal differentiation in HO-1 cells, can directly modify extracellular matrix and extracellular matrix receptor gene expression. The changes induced in these genes by these agents reflect a more normal, as opposed to the original, transformed cellular phenotype. In this context, it is also worth commenting on changes induced in tenascin expression as a consequence of treatment with the various differentiation-inducing and/or growth-suppressing agents. Tenascin is an extracellular-matrix protein expressed (or prominently expressed) in specialized embryonic tissues, cells of neuroectodermal

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origin, and tumors (63). In general, tenascin is expressed at higher levels in undifferentiated vs. differentiated tumors (63). HO-1 cells express tenascin, and its level of expression is increased by IFN- γ , alone or in combination with IFN- β , whereas its expression is reduced by treatment with IFN- β , MEZ, or RA or by continuous growth in the combination of IFN- β + MEZ or RA + MEZ. These results provide further evidence that the continuous treatment of HO-1 cells for 96 h with specific differentiation-inducing agents can result in acquisition of a more differentiated cellular phenotype by these human melanoma cells.

Studies analyzing the mechanism of growth arrest during the process of differentiation in hematopoietic cells have implicated IFN- β as an autocrine growth inhibitor important in this process (39, 64, 65). Supporting evidence for the involvement of autocrine IFN- β in the differentiation process of hematopoietic cells include (1) the ability of IFN- β antibody, but not IFN- α antibody, to partially block the reduction in c-myc mRNA and growth inhibition associated with the differentiation process; (2) the induction of interferon regulatory factor 1 (IRF-1), which is a positive transcription factor for expression of the IFN- β gene, during the myeloid differentiation process; (3) the ability of IRF-1 antisense oligomers to partially block growth inhibition associated with IL-6 and leukemia inhibitory factor induction of differentiation; and (4) the induction of type I interferon (IFN- α/β) gene expression during terminal differentiation in hematopoietic cells (39, 64, 65). A potential IFN- β autocrine loop in the induction of specific programs of reversible and irreversible differentiation in human melanoma cells is also suggested by the experiments described in this article. Reversible

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differentiation, resulting from treatment with MPA + MEZ and RA + MEZ, and terminal cell differentiation, resulting from growth in IFN- β + MEZ, results in the enhanced expression of type I interferon-responsive genes, including HLA Class I antigen, ISG-15, and ISG-54. These same gene expression changes occur in HO-1 cells treated with conditioned medium obtained from IFN- β + MEZ treated HO-1 cells. In addition, conditioned medium induces growth suppression in HO-1 cells and IFN- β antibodies partially block the induction by conditioned medium of ISG-15 in HO-1 cells (56). Attempts to quantitate IFN- β in conditioned medium from inducer-treated HO-1 cells have not been successful (66). A possible reason for the lack of quantifiable IFN- β in HO-1 inducer-treated conditioned medium could be the presence of INF- β below the sensitivity of detection of the assay system, that is, level of IFN- β below 2 U/ml. In this respect, the differentiating HO-1 system may be similar to hematopoietic cells induced to terminally differentiate by treatment with various inducers that also produce small quantities of high specific-activity autocrine IFN- β (39). Further studies are required to characterize the putative autocrine IFN- β produced by differentiating human melanoma cells and to determine its potential role in both the reversible commitment to differentiation and terminal differentiation in human melanoma cells. The present studies support the hypothesis that autocrine IFN- β may also contribute to the differentiation process in solid tumors.

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Analysis of gene expression changes resulting from exposure to IFN- β + MEZ and conditioned medium from HO-1 cells treated with these inducers suggests the presence of additional autocrine factors produced during the differentiating process in HO-1 cells. One of these

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putative autocrine factors is the previously identified melanoma growth factor termed MGSA (67). MGSA has been identified in the serum-free growth medium obtained from low-density cultures of the human malignant cell line Hs294T (67). The gene for MGSA has been cloned (68) and the deduced amino acid sequence for human MGSA is identical to that of the human "gro" cDNA isolated by Anisowicz et al. (69), now referred to as gro/MGSA. gro/MGSA is secreted by ~70% of primary cell cultures derived from human melanoma biopsies and by a majority of benign nevus cells with chromosomal abnormalities, whereas benign nevus cells with a normal karyotype are negative for MGSA production (70, 71). The level of gro/MGSA mRNA is enhanced in human melanoma cells treated with MGSA, indicating a potential autocrine function for this molecule (68); gro- α and gro- β have also been shown to be primary response genes that are induced as a result of IL-1-mediated growth arrest in human melanoma cells (72). In addition, the expression and secretion of MGSA is strongly induced in other cell types, including human endothelial cells treated with a number of agents such as IL-1, TNF, lipopolysaccharide, thrombin, or TPA (73). These observations suggest that gro/MGSA production is not restricted to human melanoma cells and, in addition to stimulating the growth of specific melanoma cells, gro/MGSA may also play a role in the inflammation process. Applicants presently demonstrate that gro/MGSA gene expression is induced in HO-1 cells during specific programs of reversible differentiation and during terminal cell differentiation. In contrast, growth arrest, without the induction of biochemical or cellular markers of differentiation, does not result in gro/MGSA induction. The ability of conditioned medium obtained from IFN- β + MEZ-treated HO-1 cells to induce gro/MGSA in naive HO-1 cells suggests that gro/MGSA may be produced

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5 during the induction of terminal cell differentiation in
HO-1 cells. At present the function of gro/MGSA (which
is structurally related to a number of additional genes,
including platelet factor-4, β -thromboglobulin,
connective tissue-activating peptide-3, and the murine KC
gene) in melanoma development is not clear. The present
data suggest, however, that in addition to its growth
stimulatory effect on human melanoma cells, gro/MGSA may
also play a role in melanoma cell differentiation.

10 In summary, the HO-1 cells culture system has been used
to analyze the molecular changes associated with the
reversible commitment to differentiation and terminal
cell differentiation in human melanoma cells. Evidence
15 is presented indicating that induction of both processes
may involve overlapping gene expression changes.
However, the magnitude of the changes and the persistence
of the changes suggest a potential involvement of defined
programs of gene expression alterations in the induction
20 and maintenance of the terminal cells differentiation
phenotype of human melanoma cells. Although their
precise roles in melanoma cell growth and differentiation
are not presently known, data are also presented that
indicate that induction of differentiation results in the
25 production of autocrine factors, including IFN- β and
gro/MGSA. Further studies are required to define the
functional significance of specific gene expression
changes and specific autocrine factors in the process of
terminal cells differentiation in human melanoma cells.
30 This information will be important in understanding the
process of melanoma development and evolution and may
result in the identification of novel target genes and
molecules that could prove useful in the therapy of this
neoplastic disease. In addition, the HO-1
35 differentiation model system appears ideally suited for

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the identification and cloning of genes involved in the induction and maintenance of loss of proliferative capacity and terminal cell differentiation in human melanoma cells.

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References for First Series of Experiments

1. Clark, W.H., Jr., The Skin. In: Rubin, E. and Farber, J.L., Eds., Pathology, Lippincott Press Inc., New York, pp. 1194-1259. 1988.
5
2. Waxman, S., Rossi, G.B., and Takaku, F., Eds., The Status of Differentiation Therapy of Cancer, Vol. 1, 45:1-422, Serono Symposia Pubs., Raven Press, New York, 1988.
10
3. Ahmed, M.A., Nielsch, U., Guarini, L., Hermo, H., Jr., and Fisher, P.B., Modulation of differentiation: a potential mechanism by which interferons induce antitumor activity. IN: Fisher, P.B., Ed., Mechanisms of Differentiation: Modulation of Differentiation by Exogenous Agents, Vol. II, CRC Press, Boca Raton, Florida, pp. 1-56, 1990.
15
20
4. Fisher, P.B. and Rowley, P.T., Regulation of growth, differentiation, and antigen expression in human tumor cells by recombinant cytokines: Applications for the differentiation therapy of human cancer. In: Waxman, S., Rossi, G.B., and Takaku, F., Eds., The Status of Differentiation Therapy of Cancer, Vol. 2, 82:201-214, Serono Symposia Pubs., Raven Press, New York, 1991.
25
5. Waxman, S., Rossi, G.B., and Takaku, F., Eds., The Status of Differentiation Therapy of Cancer, vol. 2, 82:1-451, Serono Symposia Pubs., Raven Press, New York, 1991.
30
6. Fisher, P.B., Prignoli, D.R., Hermo, H., Jr.,
35

2174354

-88-

Weinstein, I.B., and Pestka, S., Effects of combined treatment with interferon and mezerein on melanogenesis and growth in human melanoma cells, J. Interferon Res., 5:11-22, 1985.

5

7. Fisher, P.B., Hermo, H., Jr., Solowey, W.E., Dietrich, M.C., Edwalds, G.M., Weinstein, I.B., Langer, J.A., Pestka, S., Giacomini, P., Kusama, M., and Ferrone, S., Effect of recombinant human fibroblast interferon and mezerein on growth, differentiation, immune interferon binding and tumor associated antigen expression in human melanoma cells. Anticancer Res., 6:765-774. 1986.
- 10 8. Huberman, E., Heckman, C., and Langenbach, R., Stimulation of differentiated functions in human melanoma cells by tumor-promoting agents and dimethyl sulfoxide. Cancer Res., 39:2618-2624, 1979.
- 15 9. Guarini, L., Temponi, M., Edwalds, G.M., Vita, J.R., Fisher, P.B., and Ferrone, S., In vitro differentiation and antigenic changes in human melanoma cell lines. Cancer Immunol. Immunother., 20 30:363-370, 1989.
- 25 10. Guarini, L., Graham, G.M., Jiang, H., Ferrone, S., Zucker, S., and Fisher, P.B., Modulation of the antigenic phenotype of human melanoma cells by differentiation-inducing and growth-suppressing agents. Pigment Cell Res. Suppl., 2:123-131, 1992.
- 30 11. Kiguchi, K., Collart, F.R., Henning-Chub, C., and Huberman, E., Induction of cell differentiation in melanoma cells by inhibitors of IMP dehydrogenase: 35

altered patterns of IMP dehydrogenase expression and activity. Cell Growth Differ., 1:259-270, 1990.

- 5 12. Graham, G.M., Guarini, L., Moulton, T.A., Datta, S.,
Ferrone, S., Giacomini, P., Kerbel, R.S., and
Fisher, P.B., Potentiation of growth suppression and
modulation of the antigenic phenotype in human
melanoma cells by the combination of recombinant
human fibroblast and immune interferon. Cancer
10 Immunol. Immunother., 32:382-390, 1991.
- 15 13. Giovanella, B.C., Stehlin, J.S., Santamaria, C.,
Yim, S.O., Morgan, A.C., Williams, L.J., Leibovitz,
A., Fialkow, P.Y., and Mumford, D.M., Human
neoplastic and normal cells in tissue culture. I.
Cell lines derived from malignant melanomas and
normal melanocytes. J. Natl. Cancer Inst., 56:1131-
1142, 1976.
- 20 14. Reddy, P.G., Graham, G.M., Datta, S., Guarini, L.,
Moulton, T.A., Jiang, H., Gottesman, M.M., Ferrone,
S., and Fisher, P.S., Effect of recombinant
fibroblast interferon and recombinant immune
interferon on growth and the antigenic phenotype of
25 multidrug-resistant human glioblastoma multiforme
cells. J. Natl. Cancer Inst., 83:1307-1315, 1991.
- 30 15. Su, Z.-Z., Grunberger, D., and Fisher, P.B.,
Suppression of adenovirus type 5 E1A-mediated
transformation and expression of the transformed
phenotype by caffeic acid phenethyl ester (CAPE).
Mol. Carcinogenesis, 4:231-242, 1991.
- 35 16. Jiang, H., Su, Z.-Z., Datta, S., Guarini, L.,
Waxman, S., and Fisher, P.B., Fludarabine phosphate

2174354

-90-

selectively inhibits growth and modifies the antigenic phenotype of human glioblastoma multiforme cells expressing a multidrug resistance phenotype. Int. J. Oncol., 1:227-239, 1992.

5

17. Erba, H.P., Gunning, P., and Kedes, L., Nucleotide sequence of the human γ -cytoskeletal actin mRNA: anomalous evolution of vertebrate non-muscle actin genes. Nucleic Acid Res., 14:5275-5294, 1986.

10

18. Angel, P., Allegretto, E.A., Okino, S.T., Hattori, K., Boyle, W.J., Hunter, T., and Karin, M., Oncogene jun encodes a sequence-specific trans-activator similar to AP-1. Nature (London), 332:166-171, 1988.

15

19. Dalla-Favera, R., Martinotti, S., Gallo, R.C., Erikson, J., and Croce, C.M., Translocation and rearrangements of the c-myc oncogene locus in human undifferentiated B-cell lymphomas. Science, 219:963-967, 1983.

20

20. Schwarzbauer, J.W., Tamkun, J.W., Lemischka, I.R., and Hynes, R.O., Three different fibronectin mRNAs arise by alternate splicing within the coding region. Cell, 35:421-431, 1983.

25

21. Cochran, B.H., Reffel, A.C., and Stiles, C.D., Molecular cloning of gene sequences regulated by platelet-derived growth factor, Cell, 33:939-947, 1983.

30

22. Maio, M., Gulwani, B., Langer, J.A., Kerbel, R.S., Duigou, G.J., Fisher, P.B., and Ferrone, S., Modulation by interferons of HLA antigens, high

35

-91-

molecular weight-melanoma associated antigens and intercellular adhesion molecule-1 expression by cultured melanoma cells with different metastatic potential. *Cancer Res.*, 49:2980-2987, 1989.

- 5
23. Schreiner, C.L., Bauer, J.S., Danilov, Y.N., Hussein, S., Szczekan, M.M., and Juliano, R.L., Isolation and characterization of Chinese hamster ovary cell variants deficient in the expression of fibronectin receptor. *J. Cell Biol.*, 109:3157-3167, 1989.
- 10
- 15
25. Larner, A.C., Jonak, G., Cheny, Y.-S.E., Korant, B., Knight, E., and Darnell, J.E., Jr., Transcriptional induction of two genes in human cells by α -interferon. *Proc. Natl. Acad. Sci. U.S.A.*, 81:6733-6737, 1984.
- 20
26. Ryder, K., Lau, L.F., and Nathans, D., A gene activated by growth factors is related to the oncogene v-jun. *Proc. Natl. Acad. Sci. U.S.A.*, 85:1487-1491, 1988.
- 25
27. Gulcher, J.R., Nies, D.E., Marton, L.S., and Stafansson, K., An alternatively spliced region of the human hexabrachion contains a repeat of potential N-glycosylation sites. *Proc. Natl. Acad. Sci. U.S.A.*, 86:1588-1592, 1989.
- 30
28. Mark, D.V., Lu, S.D., Creasey, A., Yamamoto, R., and Lin, L., Site-specific mutagenesis of the human fibroblast interferon gene. *Proc. Natl. Acad. Sci.*
- 35

2174354

-92-

U.S.A., 81:5662-5666, 1984.

29. Kung, H.-F., Pan, Y.-C.E., Moschera, J., Tsai, K.,
Bekesi, F., Chang, M., Sugino, H., and Honda, S.,
5 Purification of recombinant human immune interferon.
Methods Enzymol., 119:435-447, 1986.
30. Rehberg, G., Kelder, B., Hoal, E.G., and Pestka, S.,
10 Specific molecular activities of recombinant and
hybrid leukocyte interferons. J. Biol. Chem.,
257:11497-11502, 1982.
31. Levy, D.E., Kessler, D.S., Pine, R., Reich, N., and
15 Darnell, J.E., Jr., Interferon-induced nuclear
factors that bind a shared promoter element
correlate with positive and negative control. Genes
Dev., 2:383-393, 1988.
32. Schindler, C., Shuai, K., Prezioso, V.R., and
20 Darnell, J.E., Jr., Interferon-dependent tyrosine
phosphorylation of a latent cytoplasmic
transcription factor. Science, 257:809-813, 1992.
33. DePinho, R.A., Schreiber-Agus, N., and Alt, F.W.,
25 Myc family oncogenes in the development of normal
and neoplastic cells. In: Vande Woude, G.F., and
Klein, G., Advances in Cancer Research, Academic
Press, San Diego, CA, pp. 1-38, 1991.
- 30 34. Torres, R., Schreiber-Agus, N., Morgenbesser, S.D.,
and DePinho, R.A., Myc and max: a putative
transcriptional complex in search of a cellular
target. Curr. Opinion Cell Biol., 4:468-474, 1992.
- 35 35. Kerr, L.D., Inoue, J.-i., and Verma, I.M., Signal

-93-

transduction: the nuclear target. Curr. Opinion Cell. Biol., 4:496-501, 1992.

- 5 36. Lachman, H.M. and Skoultchi, A.I., Expression of c-myc changes during differentiation of mouse erythroleukemia cells. Nature, 310:249-251, 1984.
- 10 37. Einat, M., Resnitzky, D., and Kimchi, A., Close link between reduction of c-myc expression by interferon and G₀/G₁ arrest. Nature, 313:597-600, 1985.
- 15 38. Yarden, A., and Kimchi, A., Tumor necrosis factor reduces c-myc expression and cooperates with interferon- γ in HeLa cells. Science, 234:1419-1421, 1986.
- 20 39. Resnitzky, D., Yarden, A., Zipori, D., and Kimchi, A., Autocrine β -related interferon controls c-myc suppression and growth arrest during hematopoietic cell differentiation. Cell, 46:31-40, 1986.
- 25 40. Resnitzky, D. and Kimchi, A., Deregulated c-myc expression abrogates the interferon- and interleukin 6-mediated G₀G₁ cell cycle arrest but not other inhibitory responses in M1 myeloblastic cells. Cell Growth Differ., 2:33-41, 1991.
- 30 41. Grief, A.E., and Westphal, H., Antisense myc sequences induce differentiation of F9 cells. Proc. Natl. Acad. Sci. U.S.A., 85:6806-6810, 1988.
42. Holt, J.T., Redner, R.L., and Nienhuis, A.W., An oligomer complementary to c-myc mRNA inhibits proliferation of HL-60 promyelocytic cells and

2174354

- 94 -

induces differentiation. Mol. Cell Biol., 8:963-973, 1988.

- 5 43. Prochowinik, E.W., Kukowska, J., and Rodgers, C., c-myc antisense transcripts accelerate differentiation and inhibit G1 progression in murine erythroleukemia cells. Mol. Cell Biol., 8:3683-3695, 1988.
- 10 44. Yokayama, K. and Imamoto, F., Transcriptional control of the endogenous myc protooncogene by antisense RNA. Proc. Natl. Acad. Sci. U.S.A., 84:7363-7367, 1987.
- 15 45. Freytag, S.O., Enforced expression of the c-myc oncogene inhibits cell differentiation by precluding entry into a distinct predifferentiation state in G₀G₁. Mol. Cell Biol., 8:1614-1624, 1988.
- 20 46. Kouzarides, T. and Ziff, E., The role of the leucine zipper in the fos-jun interaction. Nature, 336:646-651, 1988.
- 25 47. Ransone, L.J. and Verma, I.M., Nuclear proto-oncogenes Fos and Jun. Ann. Rev. Cell Biol., 6:539-557, 1990.
- 30 48. Sherman, M.L., Stone, R.M., Datta, R., Bernstein, S.H., and Kufe, D.W., Transcriptional and post-transcriptional regulation of c-jun expression during monocytic differentiation of human myeloid leukemic cells. J. Biol. Chem., 265:3320-3323, 1990.
49. Nakamura, T., Datta, R., Rubin, E., Nakamura, T.,

Hass, R., and Kufe, D., Regulation of c-jun expression during induction of monocytic differentiation by okadaic acid. Cell Growth Differ., 2:267-272, 1991.

5

50. Kharbanda, S., Datta, R., Rubin, E., Nakamura, T., Hass, R., and Kufe, D., Regulation of c-jun expression during induction of monocytic differentiation by okadaic acid. Cell Growth Differ., 3:391-399, 1992.

10

51. Yang-Yen, H.-F., Chiu, R., and Karin, M., Elevation of AP1 activity during F9 cell differentiation is due to increased c-jun transcription. New Biologist, 2:351-361, 1990.

15

52. Chiu, R., Angel, P., and Karin, M., Jun-B differs in its biological properties from, and is a negative regulator of, c-Jun. Cell, 59:979-986, 1989.

20

53. Schutte, J., Viallet, J., Nau, M., Segal, S., Fedorko, J., and Minna, J., Jun-B inhibits and c-fos stimulates and transforming and trans-activating activities of c-jun. Cell, 59:987-997, 1989.

25

54. Datta, R., Sherman, M.L., Stone, R.M., and Kufe, D., Expression of the jun-B gene during induction of monocytic differentiation. Cell Growth Differ., 2:43-49, 1991.

30

55. Lord, K.A., Hoffman-Liebermann, B., and Liebermann, D.A., Complexity of the immediate early response of myeloid cells to terminal differentiation and growth arrest includes ICAM-1, Jun-B and histone variants. Oncogene, 5:386-396, 1990.

35

-96-

57. Ben-Ze'ev, A., Rodriguez Fernandez, J.L., Baum, G.,
5 and Gorodecki, B., Regulation of cell contacts, cell
configuration, and cytoskeletal gene expression in
differentiating systems. In: Fisher, P., Ed.,
Mechanisms of Differentiation: Modulation of
Differentiation by Exogenous Agents, Vol. II, CRC
10 Press, Boca Raton, Florida, pp. 143-173, 1990.
58. Ben-Ze'ev, A., The cytoskeleton in cancer cells.
Biochem. Biophys. Acta, 780:197-212, 1985.
- 15 59. Dedhar, S., Integrins and tumor invasion.
BioEssays, 12:583-590, 1990.
60. Plantefaber, L.C. and Hynes, R.O., Changes in
integrin receptors on oncogenically transformed
20 cells. Cell, 56:281-290, 1989.
61. Giancotti, F.G., and Ruoslahti, E., Elevated levels
of the $\alpha_5\beta_1$ fibronectin receptor suppresses the
transformed phenotype of Chinese hamster ovary
25 cells. Cell, 60:849-859, 1990.
62. Pytela, R., Pierschbacher, M.D., and Ruoslahti, E.,
Identification and isolation of a 140 kd cell
surface glycoprotein with properties expected of a
30 fibronectin receptor. Cell, 40:191-198, 1985.
63. Erickson, H.P. and Bourdon, M.A., Tenascin: an
extracellular matrix protein prominent in
specialized embryonic tissues and tumors. Ann. Rev.

-97-

Cell. Biol., 5:71-92, 1989.

64. Yarden, A., Shure-Gottlieb, H., Chebath, J., Revel, M., and Kimchi, A., Autogenous production of interferon- β switches on HLA genes during differentiation of histiocytic lymphoma U937 cells. EMBO J., 3:969-973, 1984.
65. Abdollahi, A., Lord, K.A., Hoffman-Liebermann, B., and Liebermann, D.A., Interferon regulatory factor 1 is a myeloid differentiation primary response gene induced by interleukin 6 and leukemia inhibitory factor: role in growth inhibition. Cell Growth Differ., 2:401-407, 1991.
67. Richmond, A., and Thomas, H.G., Purification of melanoma growth stimulatory activity. J. Cell. Physiol., 129:375-384, 1986.
68. Richmond, A., Balentine, E., Thomas, H.G., Flaggs, G., Barton, D.E., Spiess, J., Bordoni, R., Francke, U., and Derynck, R., Molecular characterization and chromosomal mapping of melanoma growth stimulatory activity, a growth factor structurally related to β -thromboglobulin. EMBO J., 7:2025-2033, 1988.
69. Anisowicz, A., Bardwell, L., and Sager, R., Constitutive overexpression of a growth-regulated gene in transformed Chinese hamster and human cells. Proc. Natl. Acad. Sci. U.S.A., 84:7188-7192, 1987.
70. Richmond, A., Fine, R., Murray, D., Lawson, D.H.,

2174354

- 98 -

and Priest, L., Growth factor and cytogenetic abnormalities in nevus and malignant melanoma cells. J. Invest. Dermatol., 86:295-302, 1986.

- 5 71. Richmond, A. and Thomas, H.G., Melanoma growth stimulatory activity: isolation from human melanoma tumors and characterization of tissue distribution. J. Cell. Biochem., 36:185-198, 1988.
- 10 72. Rangnekar, V.V., Waheed, S., and Rangnekar, V.M., Interleukin-1 inducible tumor growth arrest is characterized by activation of cell type-specific "early" gene expression programs. J. Biol. Chem., 267:6240-6248, 1992.
- 15 73. Wen, D., Rowland, A., and Derynck, R., Expression and secretion of gro/MGSA by stimulated human endothelial cells. EMBO J., 8:1761-1766, 1989.

Second Series of Experiments

5 Molecular biological approaches for the identification
and cloning of genes displaying differential expression
in both related and different cell types have been
described (1-5). A particularly powerful procedure that
has resulted in the identification of genes
differentially expressed in diverse target cells is
subtraction hybridization (4,5). This approach has been
10 successfully used to identify genes that are specifically
expressed during progression of the transformed/malignant
phenotype (5,6), in cells undergoing growth arrest (7),
induced by specific DNA damaging agents (8), expressed
during specific stages of B cell development (9), and
15 associated with programmed cell death (10). Subtraction
hybridization is ideally suited for the identification of
rare transcripts (4,5,9,11,14), or transcripts that
exhibit small variations in expression between two cell
types (4,8,11). As described in this article,
20 subtraction hybridization is also an ideal procedure for
identifying and cloning genes that are expressed at
higher levels in cells induced to differentiate versus
uninduced parental cells. In addition, by performing
subtraction in the opposite direction, i.e.,
25 differentiation-inducer treated (Ind^+) from untreated
control (Ind^-), the present protocol can also be used to
identify genes that are suppressed during terminal
differentiation.

30 Treatment of human melanoma cells with the combination of
recombinant human fibroblast interferon (IFN- β) and the
antileukemic compound mezerein (MEZ), results in a rapid
cessation of cell growth and the induction of terminal
cell differentiation, i.e., cells remain viable, but

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they lose proliferative capacity (15-17). Terminal cell differentiation can be induced by IFN- β plus MEZ in human melanoma cells either innately sensitive or resistant to either agent used alone (15,16). In contrast, treatment of melanoma cells with either IFN- β or MEZ alone results in a reversible alteration in differentiation phenotypes in the human melanoma cell line H0-1. This system represents a valuable experimental model for determining which changes in gene expression are correlated directly with growth suppression as opposed to reversible differentiation and terminal cell differentiation. Applicants have presently developed a simple and effective subtraction hybridization protocol and used it to identify melanoma differentiation associated (*mda*) genes displaying enhanced expression in cells treated with reversible- and terminal differentiation inducing compounds. Four types of *mda* genes have been identified, including genes upregulated by both IFN- β and IFN- β plus MEZ, both MEZ and IFN- β plus MEZ, all three treatments and only the combination of IFN- β plus MEZ. This approach should prove amenable to other model systems resulting in the isolation of differentially expressed genes involved in important cellular processes.

MATERIALS AND METHODS

Cell Line and Differentiation Induction

5 The human melanoma cell line H0-1 is a melanotic melanoma derived from a 49-year-old female and was used between passage 125 and 150 (16). H0-1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (DMEM-5) at 37°C in a 5% CO₂-95% air humidified incubator. Cells were either untreated (*Ind*) or treated (*Ind*⁺) with a combination of IFN-β (2000 units per ml) and MEZ (10 ng per ml) for 2, 4, 8, 12 and 24 hr. For expression studies, H0-1 cells were untreated or treated for 12 and 24 hr with IFN-β (2000 units per ml), MEZ (10 ng per ml) or IFN-β plus MEZ (2000 units per ml plus 10 ng per ml) prior to isolation of cellular RNA and Northern blotting analysis (17).

Construction of cDNA Libraries

20 Total cellular RNA from untreated (*Ind*) and IFN-β plus MEZ treated (2,4,8,12 and 24 hr) (*Ind*⁺) samples was isolated by the guanidinium isothiocyanate/CsCl centrifugation procedure and poly(A⁺) RNA was selected following oligo(dT) cellulose chromatography (18). cDNA synthesis was performed using the ZAP-cDNA™ synthesis kit from Stratagene® (La Jolla, CA) which is based on an adaptation of the Gubler and Hoffman method (19). A primer-adapter consisting of oligo(dT) next to a unique restriction site (*Xho*I) was used for first strand synthesis. The double-stranded cDNAs were ligated to EcoRI adapters and then digested with the *Xho*I restriction endonuclease. The resultant EcoRI and *Xho*I cohesive ends allowed the finished cDNAs to be inserted into the λ ZAP II vector in a sense orientation with respect to the lac-Z promoter (20). The λ ZAP II vector

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contains pBluescript^{*} plasmid sequences flanked by bacteriophage-derived f1 sequences that facilitate in vivo conversion of the phage clones into the phagemid (20). Two cDNA libraries were constructed: a differentiation inducer-treated cDNA library (Ind⁺) (tester library); and a control uninduced cDNA library (Ind⁻) (driver library). The libraries were packaged with Gigapack II Gold Packaging Extract^{*} (Stratagene[®]) and amplified on PLK-F' bacterial cells (Stratagene[®]).

10

Preparation of Double-Stranded DNA from Ind⁺ Library

The Ind⁺ cDNA phagemid library was excised from λ ZAP^{*} using the mass excision procedure described by Stratagene[®] (La Jolla, CA) [21]. Briefly, 1×10^7 pfu of Ind⁺ cDNA library were mixed with 2×10^8 XL-1 Blue^{*} strain of Escherichia coli and 2×10^8 pfu of ExAssist helper phage in 10 mM MgSO₄, followed by absorption at 37°C for 15 min (22). After the addition of 10 ml of LB medium, the phage/bacteria mixture was incubated with shaking at 37°C for 2 hr followed by incubation at 70°C for 20 min to heat inactivate the bacteria and the λ ZAP^{*} phage particles. After centrifugation at 4000 g for 15 min, the supernatant was transferred to a sterile polystyrene tube, and stored at 4°C before use.

25

To produce double-stranded DNA, 5×10^7 pfu of the phagemids were combined with 1×10^9 SOLR strain of Escherichia coli, which are nonpermissive for the growth of the helper phages and therefore prevent coinfection by the helper phages (22), in 10 mM MgSO₄, followed by absorption at 37°C for 15 min. The phagemids/bacteria were transferred to 250 ml LB medium containing 50 μ g/ml ampicillin and incubated with shaking at 37°C overnight. The bacteria were harvested by centrifugation, and the

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double-stranded phagemid DNA was isolated by the alkali lysis method (18) and purified through a QIAGEN-tip 500 column (QIAGEN Inc., Chatsworth, CA).

5 **Preparation of Single-Stranded DNA from Control *Ind* Library**

10 The control *Ind* cDNA library was excised from lambda ZAP using the mass excision procedure described above. The phagemid (5×10^7) were combined with 1×10^9 XL-1 Blue strain of Escherichia coli in 10 mM MgSO₄, followed by absorption at 37°C for 15 min. The phagemids/bacteria were transferred to 250 ml LB medium, and incubated with shaking at 37°C for 2 hr. Helper phage VCS M13 (Stratagene®, La Jolla, CA) was added to 2×10^7 pfu/ml, and after incubation for 1 hr, kanamycin sulfate (Sigma) was added to 70 µg/ml. The bacteria were grown overnight. The phagemids were harvested and single-stranded DNAs were prepared using standard protocols (18).

20

Pretreatment of Double- and Single-Stranded DNA Prior to Hybridization

25 To excise the inserts from the vector, double-stranded DNA from the *Ind* cDNA library was digested with EcoRI and XhoI, and extracted with phenol and chloroform followed by ethanol precipitation (5). After centrifugation, the pellet was resuspended in distilled H₂O. Single-stranded DNA from *Ind* cDNA library was biotinylated using photoactivatable biotin (Photobiotin*, Sigma, St. Louis, MO) (23). In a 650 µl microcentrifuge tube, 50 µl of 1 µg/µl single-stranded DNA was mixed with 50 µl of 1 µg/µl photoactivatable biotin in H₂O. The solution was irradiated with the tube slanted on crushed ice at a distance of 10 cm from a 300 watt sun lamp for

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15 min. The DNA was further biotinylated by adding 25 μ l of photoactivatable biotin to the solution which was then exposed to an additional 15 min of irradiation as described above. To remove unlinked biotin, the reaction
5 was diluted to 200 μ l with 100 mM Tris-HCl, 1 mM EDTA, pH 9.0, and extracted 3X with 2-butanol. Sodium acetate, pH 6.5 was added to a concentration of 0.3 M, and the biotinylated DNA was precipitated with two volumes of ethanol.

10

Subtracted Hybridization and Construction of Subtracted cDNA Library

Subtraction hybridization was performed essentially as described by Herfort and Garber (24) with minor
15 modifications. In a 650 μ l siliconized microcentrifuge tube, 400 ng of EcoRI- and XhoI-digested Ind⁺ cDNA library and 12 μ g of biotinylated Ind⁻ cDNA library were mixed in 20 μ l of 0.5 M NaCl, 0.05 M HEPES, pH 7.6, 0.2% (wt/vol) sodium dodecyl sulfate and 40% deionized
20 formamide. The mixture was boiled for 5 min and incubated at 42°C for 48 hr. The hybridization mixture was diluted to 400 μ l with 0.5 M NaCl, 10 mM Tris, pH 8.0, 1 mM EDTA and then 15 μ g of streptavidin (BRL®) in H₂O was added, followed by incubation at room temperature
25 for 5 min. The sample was extracted 2X with phenol/chloroform (1:1), followed by back-extraction of the organic phase with 50 μ l of 0.5 M NaCl in TE buffer, pH 8.0. An additional 10 μ g of streptavidin was added and phenol/chloroform extraction was repeated. After
30 removal of excess chloroform by brief lyophilization, the final solution was diluted to 2 ml with TE buffer, pH 8.0, and passed through a Centricon^{*} 100 filter (Amicon; Danvers, MA) 2X as recommended by the manufacturer. The concentrated DNA solution (approximately 50 μ l) was then

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lyophilized. The subtracted cDNAs were ligated to EcoRI- and XhoI-digested and CIAP treated arms of the λ ZAP II vector and packaged with Gigapack II Gold packaging extract (Stratagene®, La Jolla, Ca). The library was
5 then amplified using the PLK-F' bacterial cell.

Screening Subtracted cDNA Library

The mass excision of the library was performed using ExAssist helper phage as described above. The SOLR
10 strain of Escherichia coli and cDNA phagemids were mixed at 37°C for 15 min and plated onto LB plates containing ampicillin and IPTG/X-gal. White colonies were chosen at random, isolated and grown in LB medium. Plasmid minireps and restriction enzyme digestions were performed
15 to confirm the presence of inserts. The inserts were isolated and used as robes for Northern blotting analysis (5,25). Total cellular RNA was prepared from H0-1 cells treated with IFN- β (2000 units/ml), MEZ (10 ng/ml), and IFN- β plus MEZ (2000 units/ml plus 10 ng/ml),
20 electrophoresed in 0.8% agarose gels and transferred to nylon membranes (Amersham, Arlington Heights, IL). Radiolabeled probes were generated by random oligonucleotide priming (25). Prehybridization, hybridization, posthybridization washes, and
25 autoradiography were performed as described (5,18,25).

Sequencing of mda genes

The mda clones were sequenced using double-stranded pBluescript* DNA as the template. DNA sequencing was
30 performed using the Sanger dideoxynucleotide method with sequenase* (United States Biochemical Corp., Cleveland, Ohio) and T3 promoter primer (GIBCO® BRL®, Gaithersburg, Md). This approach generates sequences from the 5' end of the inserts. Sequences were tested for homology to
35 previously identified sequences using the GenBank* EMBL

* Trade-mark

database and the GCG/FASTA computer program.

EXPERIMENTAL RESULTS

Subtraction hybridization represents a valuable methodology for isolating cDNA clones representing preferentially expressed mRNAs without prior knowledge of the selected gene or its encoded product (1-10). This procedure results in a substantial enrichment of differentially expressed cDNA clones and is often preferable to differential hybridization procedures using total cDNAs (4,5,11-14). A number of protocols have been reported for the generation of subtraction libraries [reviewed in 4]. The traditional approach involves hybridization of a first strand cDNA (tester) made from the mRNA of one cell type with mRNA (driver) prepared from a second cell type [or the first cell type treated with a specific gene modulating agent(s)] [7-9]. Single-stranded unhybridized cDNAs are then selected by hydroxylapatite column chromatography and they are used as templates for the synthesis of second-strand cDNA (7-9). However, this procedure has a number of limitations, including the requirement for RNA handling during hybridization, which can be problematic, and the limited quantity of cDNAs recovered following hybridization and column chromatography. Other subtraction hybridization protocols involve hybridization of cDNA and photobiotinylated RNA (23,26). Problems may still arise because of the requirement for large amounts of mRNA and from manipulation of RNA during the hybridization procedure (22). Recent improvements in subtraction hybridization utilize cDNA libraries as both tester and driver nucleic acid populations (24,27,28). By using driver sequences present in cloned forms, the newer approaches circumvent the problems associated with insufficient quantities of mRNAs or difficulties

resulting during the preparation and manipulation of mRNAs. Improvements in subtraction hybridization procedures have included: the use of phagemid subtraction hybridization (27); the use of single-stranded phagemids with directional inserts (28); and the use of double-stranded cDNA inserts as *tester* and single-stranded cDNAs as the *driver* (21).

The procedure applicants have used to construct subtraction libraries involves a modification of the protocols described by Rubenstein et al. (28) and Herfort and Garber (24). This strategy is outlined in Figure 8. Applicants' approach to subtraction library construction uses λ phage and commercially available reagents. In other similar procedures (22,27,28), the end products of subtraction hybridization are either single-stranded phagemid DNA, which is converted to double-stranded DNA, or double-stranded inserts, which are ligated to plasmid vectors. These procedures have two potentially limiting drawbacks including, the lower efficiency of bacterial transformation with plasmids versus phage infection and the need for special precautions to remove the double-stranded phagemids contaminating the *driver* single-stranded DNA preparation. By using λ page as vectors, these problems are easily avoided. The efficiency of phage infection of bacteria is high, often attaining levels of 10^9 PFU/ μ g DNA (21). In addition, problems with contaminating plasmids in the preparation are also eliminated because they will not be packaged and transfected into bacteria. This approach, therefore, results in the construction of subtraction libraries of high titer. By employing λ Uni-ZAP vectors which can be converted into phagemids by in vivo excision, the laborious work of subcloning the DNA inserts into

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plasmids is unnecessary.

cDNA libraries and subtraction libraries are prepared using the commercial ZAP-cDNA™ synthesis kit from Stratagene® (La Jolla, CA) (5). This product has several advantages for the construction of subtraction libraries. First, the XhoI adapter-primer permits the cDNA to be inserted into the vector in a unidirectional orientation. The efficiency of subtraction hybridization will be high if hybridization occurs only between complementary molecules in the different cDNA libraries instead of complementary molecules in the same cDNA library. This improved subtraction hybridization is achieved by using both single-stranded and double-stranded unidirectional cDNA libraries from each experimental condition. For construction of *mda* subtraction libraries (Figure 8), both the H0-1 control *Ind*⁺ and the IFN-β plus MEZ treated *Ind*⁺ cDNA libraries were constructed in a unidirectional manner. The efficiency of subtraction hybridization was insured by using single-stranded unidirectional *Ind*⁺ cDNA as the driver. Secondly, the bacteriophage f1 origin of replication, which is present in the λ ZAP II vector, permits excision of pBluescript II SK(-) phagemids from the bacteriophage and rescue of single-stranded DNA with the assistance of helper phage (20). The Escherichia coli strains XL-1 blue and SOLR, which are provided as part of the ZAP-cDNA™ kit, are very useful in preparing single-stranded and double-stranded phagemid DNA. The XL-1 blue strain is permissive for ExAssist helper phage growth, while the SOLR strain is nonpermissive (22). The phagemids are excised with ExAssist helper phage in XL-1 blue bacterial cells. The phagemids are then grown in SOLR bacterial cells for harvesting double-stranded DNA or in XL-1 blue cells with helper phages for harvesting

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single-stranded DNA. Using this approach, contamination of helper phage and single-stranded DNA in the double-stranded DNA preparation was minimized. Contamination could decrease the efficiency of subtraction hybridization because of the complementary binding between the single-stranded cDNA and any potentially unique sequences from the same cDNA library (tester). This could potentially result in a failure of the unique sequences to form double-stranded inserts with appropriate ends which can be ligated into the vectors. A third consideration is the commercial availability of Uni-ZAP* arms which can be used as vector for the construction of subtraction libraries. Tester inserts are released from the phagemid vector by digestion with the restriction enzyme EcoRI and XhoI. After subtraction hybridization, the remaining inserts which are in the double-stranded form because of complementary hybridization are ligated in a unidirectional manner into Uni-ZAP* arms because of the EcoRI and XhoI cohesive ends. This approach eliminates the requirement for additional vectors. The subtraction library is then converted into a phagemid library which can be easily manipulated for screening, sequencing, in vitro RNA transcription, and mutagenesis. Without the advent of well designed commercial kits, subtraction hybridization and subtraction library construction is both time and labor intensive. The procedure applicants describe in this article is straight forward and highly efficient in producing subtraction libraries.

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Employing the approaches applicants describe above, cDNA libraries from control H0-1 cells (Ind⁻) and H0-1 cells treated with the terminal differentiation inducing agents IFN- β plus MEZ (Ind⁺) have been constructed. The

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original titers of the cDNA libraries were 1.2×10^6 PFU and 1.7×10^6 PFU for the *Ind* and *Ind*⁺, respectively. The high titers obtained suggest that the cDNA libraries are representative of the mRNAs produced under the experimental conditions used. The purity of the single-stranded and double-stranded DNA was examined by digestion with the restriction endonucleases EcoRI and XhoI. Unlike double-stranded DNA, the single-stranded DNA could not be digested with the restriction endonucleases. This is demonstrated in Fig. 9, in which plasmid vector is released after digestion of double-stranded DNA but not single-stranded DNA. Four hundred ng of double-stranded DNA (tester:*IND*⁺) and 12 μ g of single-stranded DNA (driver:*Ind*) were used for subtraction hybridization. After a single-round of hybridization, the *Ind*⁺ subtraction library was constructed in Uni-ZAP XR vector with an original titer of 8 to 10×10^3 PFU. Additional rounds of subtraction hybridization resulted in a low percentage of colonies which contained inserts. This may result because of the low concentration of potentially unique sequences remaining after the first round of subtraction hybridization. This observation indicates that the subtraction hybridization protocol applicants have utilized is very efficient and the requirement for additional subtraction hybridizations may not be necessary to identify differentially expressed genes.

Screening of subtraction libraries for differentially expressed sequences can be achieved using several procedures. In a number of studies, subtraction libraries are screened using differential hybridization techniques (7, 8, 27). However, the sensitivity of this procedure is limited by the relative abundance of the

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target mRNA. The enrichment of target sequences obtained in our subtractions libraries permitted the random isolation of clones for evaluation of mRNA expression in undifferentiated H0-1 cells or H0-1 cells treated with IFN- β , MEZ or IFN- β + MEZ. After in vivo excision, bacteria containing the subtraction library were plated and randomly isolated clones were used to prepare plasmids. The EcoRI/XhoI digested cDNA inserts from these clones were then used as probes for Northern blotting analysis of mRNA expression under the different experimental conditions. Among 70 cDNA clones initially analyzed, 23 clones were found to display differences in gene expression between *Ind* and *Ind*⁺ treated H0-1 cells. As expected, subtraction of control H0-1 cDNAs from IFN- β plus MEZ treated H0-1 cDNAs results in a series of *mda* genes which displayed enhanced expression after 24 hr treatment with the inducer. These included *mda* genes which were inducible by both IFN- β and IFN- β plus MEZ, i.e. *mda*-1 and *mda*-2; by both MEZ and IFN- β plus MEZ, i.e., *mda*-3; by IFN- β , MEZ and IFN- β plus MEZ, i.e., *mda*-4; and uniquely by IFN- β and MEZ, i.e., *mda*-5 and *mda*-6 (Fig. 10). Specific *mda* genes also displayed elevated expression after 96 hr exposure to IFN- β plus MEZ (data not shown).

25

Of the six *mda* genes reported in this study, only *mda*-3 corresponds to a previously reported gene (Fig. 11). At present, 245 bp of *mda*-3 have been sequenced and this cDNA shares >99% homology with the reported sequences of pLD78 (29), pAT 464 (30,31), pAT 744 (31) and GOS19 (32-34). The pLD78 cDNA is inducible by either TPA or a T-cell mitogen, phytohemagglutinin (PHA), in human tonsillar lymphocytes (29). *mda*-3 is induced in H0-1 cells within 24 hr of treatment with MEZ, IFN- β plus MEZ and IFN- β

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plus TPA (data not shown). The sequence of the 5' flanking region of the genomic DNA encoding for the pLD78 cDNA displayed a significant homology with corresponding regions of the human interleukin 2 and immune interferon genes (29). pAT 464 and pAT 744 are inducible by TPA and PHA, with maximal induction resulting from the combination of agents, in T-cells, B-cells and the promyelocytic cell line HL-60 (31). In contrast, these cDNAs are not expressed in human fibroblasts, although as indicated in the present study a potentially similar cDNA, *mda-3*, is inducible in human melanoma. pAT 464 and pAT 744 share some critical amino acid similarity with a family of secreted factors including connective tissue activating factor III, platelet factor 4, an IFN- γ -induced factor, macrophage inflammatory protein and a factor chemotactic to neutrophils (3-10C, monocyte-derived neutrophil chemotactic factor, neutrophil-activating factor) (31). GOS19 genes are members of the "small inducible" family of genes, which exhibit similar exon-intron organizations and which encode secreted proteins with similar organization of cysteine and proline residues (32-34). The GOS19-1 mRNA is enhanced rapidly by the addition of both cycloheximide or lectin to cultured human blood mononuclear cells (32). This cDNA has sequence homology to the murine gene that encodes an inhibitory cytokine (MIP1 α /SCI) which decreases stem cell proliferation (32). In this context, GOS19-1, which is the main GOS19 gene expressed in adult T lymphocytes, may encode a homeostatic negative regulator of marrow stem cell populations. The role of *mda-3* in the process of melanoma cell growth and differentiation remains to be determined.

Studies are currently in progress to further characterize the novel *mda* genes, *mda-1*, *mda-2*, *mda-4*, *mda-5*, and *mda-*

6, and determined their expression in different stages of melanoma evolution and during the induction of growth suppression, the reversible commitment to differentiation and the induction of terminal differentiation in human melanoma cells. It should be emphasized that the cDNA clones applicants have currently analyzed represent only a small percentage of the complete subtraction library. This suggests that this subtraction library has the potential for identifying and cloning additional genes involved or associated with the chemical induction of differentiation and growth suppression in human melanoma cells. In addition, by altering the driver DNA, i.e., using combinations of cDNA libraries constructed from H0-1 cells treated singularly with IFN- β and MEZ, it should be possible to further enrich for gene(s) uniquely expressed in terminally differentiated human melanoma cells, i.e. those treated with the combinations of IFN- β + MEZ.

In summary, applicants presently describe an efficient and sensitive procedure for the production of subtraction hybridized cDNA libraries which can be used for the identification and cloning of differentially expressed genes. The basic protocol utilizes biotinylated single-stranded DNA as the driver and bacteriophage as the vector and relies on the availability of commercial reagents for construction of subtraction cDNA libraries. The usefulness of the current protocol is demonstrated by the high level of enrichment obtained from genes in the subtracted library associated with the induction of differentiation of human melanoma cells, i.e., *mda* genes. This procedure should find wide applicability for the identification and cloning of differentially expressed genes. These can include, but are not limited to, genes displaying modified expression between closely related

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cell types, between disparate cell types, in cells induced to lose proliferative ability or undergo apoptosis, in cells treated with chemotherapeutic agents, and in cells induced or committed to reversible or terminal differentiation.

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References for the Second Series of Experiments

1. Scott, M.D., Westphal, H.-H., and Rigby, R.W.J.,
Activation of mouse genes in transformed cells,
5 *Cell*, 34:557-567 (1983).
2. Davis, M.M., Cohen, D.I., Nielsen, E.A., Steinmetz,
M., Paul, W.E., and Hood, L. Cell-type specific cDNA
probes and the murine I region: The localization and
10 orientation of A_d , *Proc. Natl. Acad. Sci. USA*,
81:2194-2198 (1984).
3. Lee, S.W., Tomasetto, C., and Sager, R. Positive
selection of candidate tumor-suppressor genes by
15 subtractive hybridization, *Proc. Natl. Acad. Sci.*
USA, 88:2825-2829 (1991).
4. Schweinfest, C.W., and Papas, T.S. Subtraction
hybridization: an approach to the isolation of genes
20 differentially expressed in cancer and other
biological systems (Review), *Intl. J. Oncology*,
I:499-506 (1992).
5. Reddy, P.G., Su, Z.-Z., and Fisher, P.B.
25 Identification and cloning of genes involved in
progression of transformed phenotype. In: Adolph,
K.W., Ed., *Methods in Molecular Genetics*, Vol. I,
Academic Press, Orlando, Florida, pp. 68-102 (1993).
- 30 6. Matrisian, L.M., Bowden, G.T., Krieg, P.,
Furstenberger, G., Briand, J.-P., Leroy, P., and
Breathnach, R. The mRNA coding for the secreted
protease transin is expressed more abundantly in
malignant than in benign tumors, *Proc. Natl. Acad.*

-116-

Sci. USA, 83:9413-9417 (1986).

7. Schneider, C., King, R.M., and Philipson, L. Genese specifically expressed at growth arrest of mammalian cells, *Cell*, 54:787-793 (1988).
5
8. Fornace, A.J., Jr., Alamo, I. Jr., and Hollander, M.C. DNA damage-inducible transcripts in mammalian cells, *Proc. Natl. Acad. Sci. USA*, 85:8800-8804 (1988).
10
9. Yancopoulos, G.D., Oltz, E.M., Rathburn, G., Berman, J.E., Smith, R.K., Lansford, R.D., Rothman, P., Okada, A., Lee, G., Morrow, M., Kaplan, K., Prockop, S., and Alt, F.W. Isolation of coordinately regulated genes that are expressed in distinct stage of B-cell development, *Proc. Natl. Acad. Sci. USA*, 87:5759-5763 (1990).
15
10. Owens, G.P., Hahn, W.E., and Cohen, J.J. Identification of mRNAs associated with programmed cell death in immature thymocytes, *Mol. Cell. Biol.*, 11:4177-4188 (1991).
20
11. Fragnoli, J., Holbrook, N.J., and Fornace, A.J., Jr. Low-ratio hybridization subtraction, *Anal. Biochem.*, 187:364-373 (1990).
25
12. Frohman, M.A., Dush, M.K., and Martin, G.R. Rapid production of full-length cDNAs from rare transcripts: amplification using a single-gene specific oligonucleotide primer, *Proc. Natl. Acad. Sci. USA*, 85:8998-9002 (1988).
30
13. Timblin, C.J., Battey, G., and Kuehl, W.M.
35

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-117-

Application for PCR technology to subtractive cDNA cloning: Identification of genes expressed specifically in murine plasmacytoma cells, *Nucleic Acids Res.*, 18:1587-1593 (1990).

- 5
14. Wieland, I., Bolgar, G., Asouline, G., and Wigler, M. A method for difference cloning: Gene amplification following subtractive hybridization, *Proc. Natl. Acad. Sci. USA*, 87:2720-2724 (1990).
- 10
15. Fisher, P.B., Hermo, H., Jr., Solowey, W.E., Dietrich, M.C., Edwalds, G.M., Weinstein, I.B., Langer, J.A., Pestka, S., Giacomini, P., Kusama, M., and Ferrone, S. Effect of recombinant human fibroblast interferon and mezerein on growth, differentiation, immune interferon binding and tumor associated antigen expression in human melanoma cells, *Anticancer Res.*, 6:765-774 (1986).
- 15
- 20 16. Fisher, P.B., Prignoli, D.R., Hermo, H., Jr., Weinstein, I.B., and Pestka, S. Effects of combined treatment with interferon and mezerein on melanogenesis and growth in human melanoma cells, *J. Interferon Res.*, 5: 11-22 (1985).
- 25
17. Jiang, H., Su, Z.-Z., Boyd, J., and Fisher, P.B. Gene expression changes induced in human melanoma cells undergoing reversible growth suppression and terminal cell differentiation, *Mol. Cell. Different.*, 1:41-66 (1993).
- 30
18. Sambrook, J., Fritsch, E.F., and Maniatis, T., Eds., *Molecular Cloning: A Laboratory Manual*, 2nd edition, Cold Spring Harbor Lab. Press, Cold Spring Harbor, New York, 1989.
- 35

2174354

-118-

19. Gubler, U., and Hoffman, B.J. A simple and very effective method for generating cDNA libraries, *Gene*, 25:263-269 (1983).
- 5 20. Short, J.M., Fernandez, J.M., Sorge, J.A., and Huse, W.D. λ ZAP: A bacteriophage λ expression vector with in vivo excision properties, *Nuc. Acids Res.*, 16:7583-7600 (1988).
- 10 21. Short, J.M., and Sorge, J.A. In vivo excision properties of bacteriophage λ ZAP expression vector, *Meth. Enz.*, 216:495-508 (1992).
- 15 22. Hay, B., and Short, J.M. ExAssistTM helper phage and SOLRTM for lambda ZAP II excision, *Stratagies*, 5:16-18 (1992).
- 20 23. Sive, H.L., and St. John, T. A simple subtraction technique employing photoactivable biotin and phenol extraction, *Nuc. Acids Res.*, 16:10937 (1988).
- 25 24. Herfort, M.R., and Garber, A.T. Simple and Efficient subtractive hybridization screening, *BioTechniques*, 11(5):598-603 (1991).
- 30 25. Jiang, H., Su, Z.-Z., Datta, S., Guarini, L., Waxman, S., and Fisher, P.B. Fludarabine phosphate selectively inhibits growth and modifies the antigenic phenotype of human glioblastoma multiforme cells expressing a multidrug resistance phenotype, *Intl. J. Oncology*, 1:227-239 (1992).
26. Swaroop, A., Xu, J., Agarwal, N., and Weissman, S. A simple and efficient cDNA subtraction procedure:

isolation of human retina-specific cDNA clones, *Nuc. Acids Res.*, 19:1954 (1991).

- 5 27. Duguid, J.R., Rohwer, R.G., and Seed, B. Isolation of cDNAs of scrapie-modulated RNAs by subtractive hybridization, *Proc. Natl. Acad. Sci. USA*, 85:5738-5742 (1988).
- 10 28. Rubenstein, J.L.R., Brice, A.E.J., Claranello, R.D., Denney, D., Proteus, M.H., and Usdin, T.B. Subtractive hybridization system using single-stranded phagemids with directional inserts, *Nuc. Acids Res.*, 18:4833-4842 (1990).
- 15 29. Obaru, K., Fukuda, M., Maeda, S., and Shimada, K. A cDNA clone used to study mRNA inducible in human tonsillar lymphocytes by a tumor promoter, *J. Biochem.*, 99:885-894 (1986).
- 20 30. Irving, S., June, C.H., Zipfel, P.F., Siebenlist, U., and Kelly, K. Mitogen-induced genes are subject to multiple pathways of regulation in the initial stages of T-cell activation, *Mol. Cell. Biol.*, 9:1034-1040 (1989).
- 25 31. Zipfel, P.F., Blake, J., Irving, S.G., Kelly, K., and Siebenlist, U. Mitogenic activation of human T cells induces two closely related genes which share structural similarities with a new family of secreted factors, *J. Immunol.*, 142:1582-1590 (1989).
- 30 32. Blum, S., Forsdyke, R.E., and Forsdyke, D.R. Three human homologs of a murine gene encoding an inhibitor of stem cell proliferation, *DNA and Cell Biol.*, 9:589-602 (1990).
- 35

-120-

33. Irving, S., Zipfel, P.F., Blake, J., McBride, O.W.,
Morton, C.C., Burd, P.R., Siebenlist, U., and Kelly,
K. Two inflammatory mediator cytokine genes are
closely linked and variably amplified on chromosome
17q, *Nucleic Acids Res.*, 18:3261-3270 (1990).

5

34. Nakao, M., Nomyama, H., and Shimada, K. Structures
of human gene coding for cytokine LD78 and their
expression, *Mol. Cell Biol.*, 10:3646-3658 (1990).

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Third Series of Experiments

5 Development of malignant melanoma in humans, with the
exception of nodular type melanoma, is a progressive
process involving a discrete series of well defined
stages. Although the focus of intensive scientific
scrutiny, the genetic elements controlling melanocytic
conversion into the various stages of the evolving
10 melanoma have not been identified. In addition, no
consistently effective therapy is currently available to
treat metastatic melanoma. The long-term goal of the
present proposal is to define the genes regulating
melanoma growth, differentiation and progression. This
15 information could prove valuable in elucidating potential
targets for therapeutic intervention.

Tumor progression in melanocytes is associated with
altered patterns of normal melanocytic differentiation.
Chemical induction of terminal differentiation in tumor
20 cells represents a useful approach for reversing the
negative prognosis associated with specific neoplasms.
Recent studies indicate that the specific combination of
recombinant human fibroblast interferon (IFN- β) and the
antileukemic compound mezerein (MEZ) can reprogram human
25 melanoma cells to undergo terminal differentiation, i.e.,
cells retain viability but they irreversibly lose
proliferative capacity. In contrast, application of
comparable doses of IFN- β or MEZ alone to human melanoma
cells results in a reversible commitment to
30 differentiation, i.e., removal of the inducing agent
results in the resumption of cell growth and the loss of
specific differentiation-associated properties.

35 Subtraction hybridization is used to identify the
genotypic changes associated with induction of terminal

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differentiation in human melanoma cells. Using this approach, cDNAs displaying enhanced expression in melanoma cells induced to terminally differentiate versus untreated melanoma cells have been identified. Partial sequence analysis of these differentially expressed cDNAs, tentatively called melanoma differentiation associated (*mda*) genes, indicate that they consist of both known and previously unidentified genes. Specific *mda* genes may represent novel genetic elements involved in tumor cell growth and/or commitment of cells to the melanocyte lineage.

The specific aims of this proposal are to characterize and determine the functional roles of the *mda* genes in melanoma growth, differentiation, and progression. With these aims in mind studies will be conducted to:

- 1) Determine the pattern and regulation of expression of the *mda* genes in melanocytes, nevi, radial growth phase melanoma, vertical growth phase melanoma and metastatic melanoma cells;
- 2) Analyze the relationship between *mda* gene expression and the induction of reversible commitment to differentiation, growth suppression without the induction of differentiation, DNA damage and stress responses and induction of terminal differentiation in human melanoma and other model differentiation systems;
- 3) Isolate full-length cDNAs of *mda* genes that may be involved in melanoma differentiation or progression and directly determine their potential functional role in differentiation

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and progression of human melanoma;

- 5 4) Isolate and characterize the promoter region of appropriate *mda* genes and analyze their regulation in human melanocytes, nevi and melanoma.

10 Experimental strategies designed to activate genes mediating a loss of proliferative capacity and the reprogramming of melanoma cells to terminally differentiate, may represent novel approaches for effective therapeutic intervention in metastatic melanoma. Elucidation of the function of the cloned *mda* genes should provide molecular insights into the process
15 of melanoma differentiation and progression. In addition, specific *mda* genes may represent targets of clinical interest which can be exploited for suppressing the growth of metastatic melanoma and other tumorigenic cell types.

20

Background and Significance

25 Malignant melanoma epitomizes the process of tumor progression and emphasizes the selective nature of the metastatic phenotype and the growth dominant properties of metastatic cells (rev. 1 to 3). Of the numerous types of cancer developing in North American populations, melanoma is increasing at the fastest rate and it is estimated that as many as 1 in 100 currently born
30 children may eventually develop superficial spreading type melanoma (3,4). Although melanoma is readily curable at early stages, surgical and chemotherapeutic interventions are virtually ineffective in preventing metastatic disease and death in patients with advanced
35 stages of malignant melanoma. These observations

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emphasize the need for improved therapeutic approaches to more efficaciously treat patients with metastatic melanoma.

5 Development of malignant melanoma in humans, with the exception of nodular type melanoma, consists of a series of sequential alterations in the evolving tumor cells (rev. 1-4). These include conversion of a normal melanocyte into a common acquired melanocytic nevus
10 (mole), followed by the development of a dysplastic nevus, a radial growth phase (RGP) primary melanoma, a vertical growth phase (VGP) primary melanoma and ultimately a metastatic melanoma. As indicated above, although readily treatable during the early stages of
15 development even during the VGP if the lesion is $\leq$ 0.76-mm thick, currently employed techniques are not very effective (<20% survival) in preventing metastatic spread and morbidity in patients with VGP lesions > 4.0-mm thickness. This experimental model system is ideally
20 suited to evaluate the critical genetic changes that mediate both the early and late phases of melanoma evolution.

A less toxic approach to cancer therapy involves a
25 process termed differentiation therapy (5-9). Two premises underlie this therapeutic modality. (A) Many neoplastic cells display aberrant patterns of differentiation resulting in unrestrained growth; and (B) Treatment with the appropriate agent(s) can result in the
30 reprogramming of tumor cells to lose proliferative capacity and become terminally differentiated. Intrinsic in this hypothesis is the assumption that the genes that mediate normal differentiation in many tumor cells are not genetically defective, but rather they fail to be
35 appropriately expressed. The successful application of

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differentiation therapy in specific instances may result because the appropriate genes inducing the differentiated phenotype become transcriptionally activated resulting in the production of appropriate gene products required to induce terminal cell differentiation. Applicants have tested this hypothesis using human melanoma cells (10-14). Treatment of human melanoma cells with the combination of recombinant human fibroblast interferon (IFN- β) and the antileukemic compound mezerein (MEZ) results in a rapid cessation of growth, an induction of morphological changes, an alteration in antigenic phenotype, an increase in melanin synthesis and an irreversible loss in proliferative capacity, i.e., terminal cell differentiation (10,11,14). IFN- β plus MEZ effectively induce terminal differentiation in human melanoma cells innately resistant to the antiproliferative effect of either agent used alone (10). In contrast, IFN- β or MEZ applied alone induce a number of similar biochemical and cellular changes in human melanoma cells, however, these changes are often reversible following removal of the inducing agent, i.e., reversible commitment to differentiation (10,14). Although the effect of IFN- β plus MEZ toward normal human melanocytes has not been reported, Krasagakis et al. (15) did determine the effect of IFN- β plus TPA (which was present in the melanocyte growth medium) on the growth of normal human melanocytes. MEZ shares a number of in vitro properties with TPA, including its ability to replace TPA for the growth of normal melanocytes, to activate protein kinase C and to modulate cell differentiation (14,16). In contrast to TPA, however, MEZ is a very weak tumor promoter when substituted for TPA in the initiation-promotion model of carcinogenesis on mouse skin, although it is quite potent during the second phase of tumor promotion (17,18). When normal

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melanocytes are grown under optimal growth conditions, including TPA, cholera toxin, isobutylmethylxanthine and fetal bovine serum, even high doses (10,000 units/ml) of leukocyte (IFN- α), fibroblast (IFN- β) or immune (IFN- γ) interferon does not inhibit growth (15). In contrast, when grown in modified melanocyte medium not containing TPA and resulting in reduced growth potential, only IFN- β significantly inhibits proliferation. When tested in serum-free medium, all three interferon preparations are growth inhibitory toward the SKMel-28 human melanoma cell line, with IFN- β again being the most growth-suppressive (15). IFN- β has been shown to be more growth suppressive than IFN- α toward several additional human melanomas grown in serum containing medium (10). These results support the hypothesis that IFN- β may be a negative-regulator of melanocyte proliferation and malignant transformation results in an increased sensitivity to interferons (10,15). In the case of TPA, it is an obligatory requirement for the in vitro growth of normal melanocytes, whereas TPA and MEZ are growth inhibitory toward many human melanoma cells (14-16,19,20).

Melanoma represents a useful experimental model to analyze the process of tumor progression (rev 1-3). Cell culture systems are available that permit the growth of normal melanocytes, nevi and melanoma cells representing different stages of tumor progression (1-3,20-25). Analyses of the properties of cells of the melanocyte lineage indicate a number of traits that allow the different stages in melanoma evolution to be distinguished. These include: (a) morphology;; (b) life span in culture; (c) chromosomal abnormalities; (d) anchorage-independent growth; (e) tumorigenicity; (f) expression of HLA-DR (class II HLA antigens) and

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intercellular adhesion molecule-1 (ICAM-1) antigens; (g) response to the tumor promoting agent 12-0-tetradecanoyl-phorbol-13-acetate (TPA); (h) growth factor independence in vitro; (i) autocrine production of basic fibroblast growth factor (bFGF) and (j) growth inhibition by cytokines (rev 2,21,25). A limitation of the melanoma progression model, however, is the inability to obtain from the same patient who has developed a primary RGP or an early VGP melanoma (less than 0.76 mm in thickness), a genetically related more progressed melanoma. Recent studies by Dr. Kerbel and colleagues (25) suggest that by appropriate manipulation (use of matrigel) and tumor selection in nude mice, it may be possible to spontaneously progress early-stage, non- or poorly tumorigenic (in nude mice) human melanoma cell lines to a more progressed tumorigenic and metastatic state. In addition, Dr. Albino and colleagues (20) demonstrated that normal human melanocytes could be progressed to a complete melanoma phenotype and genotype following infection with a retrovirus containing the viral Ha-ras oncogene. Transformed melanocytes acquired the full spectrum of melanoma properties and displayed the same cytogenetic changes occurring during melanoma development in vivo (20). These cell lines will prove useful for evaluating the biochemical and genetic changes involved in melanoma progression. In summary, the ability to clearly define specific components of melanoma evolution will provide a valuable experimental model to define the genotypic changes mediating tumor progression.

The critical genomic changes that mediate melanoma development and progression remain to be defined. Recent studies have addressed the potential relationship between the expression of specific oncogenes, growth factor genes

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(in addition to basic fibroblast growth factor (bFGF)), growth factor receptor genes, protease genes and early response genes and melanoma progression (26-30). Using a panel of metastatic melanoma cell lines, steady state mRNA transcripts for several growth factors (bFGF, platelet-derived growth factor (PDGF)-A, PDGF-B, transforming growth factor (TGF)- β_1 , TGF- α , melanoma growth-stimulating activity (MGSA; also called gro), interleukin (IL-1 α and IL-1 β) and early response (*c-fos*, *c-jun* and *jun-B*) genes have been found (27, 28). All of the metastatic melanoma cell lines expressed the bFGF gene and the majority of metastatic melanoma expressed *c-fos*, *c-jun* and *jun-B* in both serum-free and serum containing medium. With respect to the other growth factor genes tested, each metastatic melanoma displayed a pattern of expression that was specific and different (27). In contrast, two strains of normal melanocytes expressed TGF- β_1 but not bFGF, PDGF, TGF- α or MGSA mRNA at detectable levels (27). Although metastatic melanoma and normal melanocytes express *c-fos*, *c-jun* and *jun-B*, the expression of these transcripts in normal melanocytes was dependent on the presence of growth-promoting agents in the medium (28). In contrast, different levels of the early response genes were observed in metastatic melanoma cells grown in the presence or absence serum (28). In general, an increase in *jun-B* and *c-fos* RNA transcripts and a decrease in *c-jun* RNA transcripts were observed in metastatic melanomas compared to neonatal melanocytes (28). The relevance of these differences in early response gene expression in metastatic melanomas compared to neonatal melanocytes remains to be determined. In a recent study, Albino et al. (30) used PCR to determine the level of RNA transcripts for 11 different growth factors in 19 metastatic human melanoma cell lines and 14

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normal human foreskin melanocyte cell lines. Transcripts for TGF- β_2 (19 of 19), TGF- α (18 of 19) and bFGF (19 of 19) were found in metastatic melanoma but not in the normal melanocytes. In contrast, TGF- β_1 and TGF- β_3 were expressed in both metastatic melanoma and normal melanocytes. The significance of these changes to melanoma progression is not apparent. These results suggest, however, that the differential expression of specific genes, i.e., bFGF, TGF- β_2 , TGF- α and possibly early response genes, may contribute to or may be directly related to the metastatic melanoma phenotype.

On the basis of genetic linkage analysis of familial melanoma, cytogenetic analysis, and various molecular techniques (including RFLP analysis to identify LOH in tumor DNA samples and microcell gene transfer procedures) it is now apparent that nonrandom changes in genes on chromosomes 1, 6, 7 and 9 may contribute to the etiology of human melanoma (31-41). At this stage of analysis, at least 5 genes, mapping to chromosomes 1, 6, 7 and 9, appear to contribute to the development of malignant melanoma, and extensive tumor heterogeneity also implicates additional loci as contributors to the malignant phenotype (rev. 38,41). A proposed model of tumor progression from melanocyte to metastatic melanoma suggests that alterations in chromosome 1 and 9 are early events in melanoma progression, whereas changes in chromosome 6 and 7 represent later stages of tumor progression (38,41). A direct demonstration of the suppressive role of chromosome 6 in human melanoma has recently been demonstrated (37). Employing microcell mediated gene transfer, a normal human chromosome 6 was inserted into human melanoma cells (UACC-903) and shown to suppress transformed properties in vitro and

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tumorigenic potential in nude mice (37). Dr. Welch and colleagues have also demonstrated that insertion of a normal human chromosome 6 into the C8161 human melanoma cell line results in a suppression of metastatic potential, but not tumorigenic potential (42). The apparent discrepancy between the results of Trent et al. (37) and Welch et al. (42) may relate to differences between UACC-903 and C8161 cells. C8161 cells exhibit both tumorigenic and metastatic properties in nude mice, whereas UACC-903 cells are tumorigenic but not metastatic in nude mice. This difference might mask the presence of a metastasis suppressor on chromosome 6 or alternatively might suggest that chromosome 6 contains both a tumor and a metastatic suppressor gene. The chromosome 6 containing C8161 cells have also been found to differ from parental C8161 cells in their biological response and in gene expression after treatment with IFN- β plus MEZ (43). Further studies are required to determine if a similar suppression of transformed and tumorigenic properties can be induced by reintroduction by microcell mediated transfer of chromosomes 1, 7 and/or 9 into melanoma cells containing abnormalities in these chromosomes. Similarly, the mechanism by which the putative melanoma suppressor gene(s) on chromosome 6, as well as suppressor gene(s) located on additional chromosomes, exert their effects on human melanoma cells and how these genes regulate tumor progression remain to be determined.

Recent studies have demonstrated the existence of at least 23 IFN- α genes and pseudogenes, all of which reside proximal to the IFN- β gene that is located on locus 9p (9p22-13) (44,45). Nonrandom alterations in specific loci on chromosome 9 appear to be an early event in melanoma evolution. It is intriguing, therefore, that

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90% (9/10) of informative melanoma DNAs have shown a reduction for one to five of the loci tested in the same region as the IFN- α/β gene (41) including a 2 to 3 megabase region on 9p21 in which a putative melanoma tumor-suppressor gene appears to be located (40). Similarly, homo- or homozygously deleted α - and β -interferon genes have been found in human acute lymphoblastic leukemias and human malignant gliomas (44-47). This observation is interesting, since interferons display antiproliferative activity toward both human melanoma and lymphoblastic leukemic cells and can be viewed therefore as tumor suppressor proteins (rev. 13). This data is compatible with the hypothesis that a tumor suppressor locus for both melanoma and leukemia is located on chromosome 9 and tumor suppression may in specific cancers involve alterations in the interferon gene region.

The mechanism by which the combination of IFN- β + MEZ induces a rapid irreversible inhibition in cellular proliferation and terminal differentiation in human melanoma cells remains to be determined. Since actinomycin D and cycloheximide can inhibit the induction of morphologic changes, growth suppression and the induction of differentiation in H0-1 cells induced by IFN- β + MEZ (49), transcriptional activation or suppression of specific gene(s) following treatment with these agents may be the primary determinants of induction of differentiation. A modified subtraction hybridization procedure was used to identify and characterize the critical genes that mediate and which are associated with the chemical induction (49). Using this approach, a series of cDNAs have been identified, termed melanoma differentiation associated (mda) genes, which display enhanced expression in terminally differentiated human

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melanoma cells (49). Specific cDNAs have been identified which represent novel genes, i.e., their sequences have not been described previously in any of the DNA data bases. By using appropriate sense and anti-sense oligomers and expression constructs, studies will be conducted to determine the functional role of the *mda* genes in melanoma growth, differentiation and progression. In addition, by employing human melanoma cells representing specific stages in melanocytic evolution to metastatic melanoma it will also be possible to address the relationship between states of tumor progression and susceptibility to induction of terminal differentiation. An understanding of the process of terminal cell differentiation and the function of the *mda* genes could prove useful in defining the molecular basis of melanoma progression and in designing improved strategies for the therapy of malignant melanoma and other cancers.

20 A. Induction of Terminal Differentiation in Human Melanoma Cells by IFN- β plus MEZ.

As discussed in Background and Significance, a hallmark of many cancers is an inability to undergo normal programs of cellular differentiation. If this assumption is correct, and if the genetic machinery of the tumor cells could be reprogrammed to regain their commitment to normal differentiation, then appropriate external stimuli could be employed to induce a loss of proliferative capacity and terminal differentiation (5-14). In studies designed to directly test this hypothesis, applicants have successfully induced terminal differentiation in human melanoma cells with the combination of recombinant IFN- β and the antileukemic compound mezerein (MEZ) (10,11,14). In contrast, the combination of recombinant

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leukocyte interferon (IFN- α) and MEZ resulted in a potentiation of growth suppression, but terminal differentiation was not induced, i.e., treated cells retained proliferative capacity (10). The combination of IFN- β plus MEZ was effective in inducing terminal differentiation in human melanoma cells relatively resistant or sensitive to the growth suppressive effects of either agent employed alone (10). Induction of terminal differentiation in the human melanoma cell line H0-1 by continuous exposure for 4 or 7 days to IFN- β plus MEZ was associated with: (a) a rapid, within 24 hr, inhibition in proliferation (Fig. 12) (10,11); (b) a profound alteration in cellular morphology (treated cells displayed dendrite-like processes) (Fig. 13) (10); and (c) an induction (in melanotic melanoma) or an increased synthesis (in melanotic melanoma) of melanin, a marker of melanoma cell differentiation (10). By employing varying doses of IFN- β and MEZ and different treatment schedules (24 hr, 4 days and/or 7 days), it has been possible to separate the chemical-induction of melanoma differentiation into three stages. These include an early completely reversible-induction phase (low doses of inducing agents for 4 or 7 days), a late partially reversible-induction phase (higher doses of inducing agents for 4 days), and an irreversible terminal-differentiation phase (specific doses of inducing agents for 24 hr, 4 days or 7 days)) (10-14).

In contrast to IFN- β plus MEZ that induces an irreversible loss in proliferative capacity and terminal differentiation in the human melanoma cell line H0-1, the combination of IFN- β plus IFN- γ induces enhanced growth suppression without terminal differentiation (12, 14, 50). In addition, IFN- β plus IFN- γ also fail to induce an increase in melanin synthesis in H0-1 cells (50).

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When treated with trans retinoic acid (RA), both melanin levels and tyrosinase levels are increased in H0-1 cells, but growth is not suppressed (14,51). Exposure to 3 μ M mycophenolic acid (MPA) for 96 hr results in growth inhibition, morphologic changes, enhanced melanin synthesis and enhanced tyrosinase activity in H0-1 cells (14, 51). However, these affects are reversible when H0-1 cells treated with 3 μ M MPA for 4 days are then grown in the absence of MPA for an additional 7 days (14). These results suggest that at the dose- and time-interval used, MPA (alone or in combination with MEZ) induces a reversible-induction of differentiation in H0-1 cells and not terminal differentiation. The effect of different agents on growth and the properties of H0-1 cells is summarized in Table 2 (14).

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TABLE 2

	Experimental Conditions ^a	Morphology changes ^b	Melanin Synthesis ^c	Tyrosinase activity ^d
5	RA (2.5 μ M)	-	1+	2+
	MPA (3.0 μ M)	+	2+	3+
10	MEZ (10 ng/ml)	+	1+	NT
	IFN- β (2000 U/ml)	-	1+	NT
15	IFN- γ (2000 U/ml)	-	-	NT
	RA + MEZ (2.5 μ M + 10 ng/ml)	+	NT	NT
20	MPA + MEZ (3.0 μ M + 10 ng/ml)	+	NT	NT
25	IFN- β + IFN- γ (1000 U/ml + 1000 U/ml)	-	1+	NT
30	IFN- β + MEZ (2000 U/ml + 10 ng/ml)	+	4+	NT

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TABLE 2 (Continued)

	Experimental conditions ^a	Growth Suppression (reversible) ^c	Terminal cell differentiation ^f
5	RA (2.5 μ M)	-	-
10	MPA (3.0 μ M)	-	-
	MEZ (10 ng/ml)	1+	-
15	IFN- β (2000 U/ml)	3+	-
	IFN- γ (2000 U/ml)	2+	-
20	RA + MEZ (2.5 μ M + 10 ng/ml)	1+	-
25	MPA + MEZ (3.0 μ M + 10 ng/ml)	3+	-
30	IFN- β + IFN- γ (1000 U/ml + 1000 U/ml)	4+	-
35	IFN- β + MEZ (2000 U/ml + 10 ng/ml)	4+ ^g	+
40	-----		
45	*H0-1 cells were grown for 96 hr or for 6 or 7 days (with medium changes after 3 or 4 days) in the presence of the agents indicated. For morphology, cells grown for 96 hr in the test agent were observed microscopically. For melanin synthesis, results are for 6 day assays for RA and MPA (51) or 7 day assays for MEZ, IFN- β , IFN- γ , IFN- β + IFN- γ and IFN- β + MEZ (10,50). For tyrosinase assays, results are for 6 day assays for RA, MPA and MEZ (51). Growth suppression (reversible and terminal cell differentiation) assays, refer to cultures treated with the indicated compound(s) for 96 hr prior to cell number determination, or treated for 96 hr and then grown for 2 weeks (with medium changes every 4 days) in the absence of compound prior to cell number determination.		
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^bMorphology changes refer to the development of dendrite-like processes 96 hr after growth in the indicated compound. + = presence of dendrite-like processes; - = no dendrite-like processes.

^cMelanin assays were determined as described in refs. 10,50,51. Results are expressed as relative increases based on separate data presented in refs. 10,50,51. N.T. = not tested.

^dTyrosinase assays were performed as described in ref. 11. Relative increases (of a similar magnitude) were found for RA, MPA and MEZ after 6 days exposure to these agents (51). N.T. = not tested.

^eReversible growth suppression indicates resumption of cell growth after treatment with the indicated compound(s) for 96 hr, removal of the test agent and growth for 14 days in compound(s) free medium. Further details can be found in ref. 14. The degree of initial 96 hr growth suppression is indicated as: - = no significant change in growth (< 10% reduction in growth in comparison with untreated control cultures); 1+ = ~30% reduction in growth in comparison with untreated control cultures; 2+ = ~40% reduction in growth in comparison with untreated control cultures; 3+ = ~50 to 60% reduction in growth in comparison with untreated control cultures; 4+ = ~80% reduction in growth in comparison with untreated control cultures.

^fThe combination of IFN- β + MEZ results in irreversible growth suppression.

^gTerminal cell differentiation indicates the loss of proliferative capacity after treatment with the indicated compound(s) for 96 hr, removal of the test agent and growth for 14 days in compound(s) free medium. Further details can be found in (14).

The studies briefly described above indicate that changes in growth, morphology, melanin synthesis and tyrosinase activity can be dissociated from the induction of terminal differentiation in H0-1 melanoma cells. However, the irreversible loss of proliferative capacity and terminal differentiation resulting from treatment with IFN- β plus MEZ appear to be correlated phenomena. Employing the various agents described above it will be

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possible to determine which gene expression changes are related to the various components of the differentiation process in human melanoma cells.

5 Monoclonal antibodies (MAbs) have recently been developed which recognize a series of hnRNP proteins, designated P2Ps, which display a marked reduction in both 3T3T cells and human keratinocytes induced to terminally differentiate (52,53). In contrast, P2Ps are present in
10 cells that have retained the ability to traverse the cell cycle, including cells reversibly growth arrested. A loss of P2Ps is also observed in cells that have irreversibly lost proliferative potential as a consequence of senescence, as well as induction of
15 terminal differentiation (52,53). In contrast, 3T3T cells transformed by SV40 do not undergo the terminal step of differentiation and these cells also do not show a suppression of P2P expression (52). These results support the concept that P2Ps may be directly linked to
20 proliferative capacity of cells and may prove useful as a general marker for terminal cell differentiation. In collaboration with Dr. Robert E. Scott (University of Tennessee Medical Center, Memphis, TN) applicants have begun to determine the level of P2Ps in human melanoma
25 cells induced to terminally differentiate by exposure to IFN- β plus MEZ and MPA plus MEZ (Fig. 14). When induced to terminally differentiate, a reduction in P2Ps was apparent in several independent human melanoma cell lines (data from F0-1 human melanoma cells is shown in Fig.
30 14). In contrast, employing human melanocytes immortalized by the SV40 T-antigen gene (54), IFN- β plus MEZ did not induce terminal differentiation or a reduction in P2Ps, whereas MPA plus MEZ resulted in a loss of proliferative capacity and a reduction in P2Ps.
35 In both F0-1 and FM516 SV cells, treatment with IFN- β ,

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MEZ or MPA alone did not reduce P2Ps even though growth was suppressed (data not shown). Induction of terminal differentiation by IFN- β + MEZ in HO-1 cells resulted in a loss of P2Ps, whereas either agent employed alone did not reduce P2P levels (data not shown). Although these results are preliminary, they suggest that the chemical induction of terminal differentiation and the irreversible loss of proliferative capacity in human melanoma cells is associated with a reduction in P2Ps.

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B. Gene Expression Changes induced in Human Melanoma Cells Displaying Reversible Growth Suppression, Reversible Commitment to Differentiation and Terminal Cell Differentiation.

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Applicants have begun to determine the spectrum of gene expression changes associated with growth suppression, morphologic alterations, increased melanin synthesis, enhanced tyrosinase activity and/or induction of terminal differentiation in human melanoma cells (12-14). The agents applicants have chosen result in reversible growth suppression, induction of melanin synthesis, morphologic alterations, enhanced tyrosinase activity, induction of a reversible commitment to differentiation or terminal differentiation with a concomitant loss of proliferative capacity in HO-1 melanoma cells (Table 2). The genes applicants have currently analyzed include: early response genes (c-fos, c-myc, c-jun, jun-B, jun-D and gro/MGSA) (14); interferon stimulated genes (ISG-15, ISG-54, HLA Class I and HLA Class II) (13, 14); cell adhesion molecules (P-cadherin, E-cadherin, N-cadherin and N-CAM) (14); extracellular matrix genes (fibronectin (FIB) and tenascin) (12, 14); cell surface proteoglycans/matrix receptors (syndecan, β_1 integrin

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(major FIB receptor subunit), α_5 integrin (major FIB receptor subunit)) (14); cytoskeleton genes (tropomyosin-1, γ -actin and β -actin) (14); and a housekeeping gene (GAPDH) (14). Using the gene probes indicated above, no unique gene expression change was found which only occurred in terminally differentiated H0-1 cells. These results indicate that commitment to differentiation and terminal differentiation in H0-1 melanoma cells is associated with specific patterns of overlapping gene expression changes. As will be discussed below, an interesting change in gene expression observed in both H0-1 cells committed to differentiate and induced to terminally differentiate was the induction and enhanced expression of type I interferon responsive genes and the gro/MGSA gene. These results have led to the hypothesis that specific autocrine feedback loops may contribute or are associated with the differentiation process in human melanoma cells.

20 C. Changes in Cell Cycle and Early Immediate Response Genes During the Induction of Terminal Differentiation in Human Melanoma Cells.

As discussed above, treatment of H0-1 cells with the combination of IFN- β + MEZ results in growth suppression that is apparent by 24 hr following exposure to these inducing agents (Fig. 12) (10, 14). This system has been used to evaluate the effects of the different inducers, alone and in combination, on the expression of cell cycle regulated genes, including cdc2, cyclin A, cyclin B, histone 1, histone 4, proliferative cell nuclear antigen (PCNA), c-myc, p53 and Rb. These studies can be summarized as follows: (a) A reduction in cdc2 and histone 1 was apparent under all treatment conditions.

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This effect was observed after 24 hr and was most dramatic in cells treated for 96 hr; (b) *c-myc* expression was marginally decreased by 24 hr treatment with MEZ and IFN- β + MEZ, whereas significant suppression was observed by 96 hr especially in IFN- β + MEZ treated H0-1 cells; (c) Both PCNA and p53 gene expression was reduced only in cells treated with IFN- β + MEZ; and (d) Rb levels remained unchanged following any of the treatment protocols. In the case of *cdc2* and histone 1, IFN- β + MEZ resulted in a decreased rate of transcription of these genes. Similarly, IFN- β + MEZ decreased the stability of the *cdc2* and histone 1 mRNAs. Analysis of cell cycle distribution by FACS analysis indicated that both MEZ and IFN- β + MEZ reduced the number of H0-1 cells undergoing DNA synthesis by 48-hour treatment. The most effective inhibitor of DNA synthesis in H0-1 cells was IFN- β + MEZ. These results indicate that the induction of terminal differentiation in H0-1 cells by IFN- β + MEZ is associated with a suppression in specific cell cycle related genes that occur at both a transcriptional and a postranscriptional level.

c-fos, *c-jun* and *jun-B* expression were superinduced in H0-1 cells treated with cycloheximide and IFN- β + MEZ, indicating that these genes are immediate early response genes. Differences in the temporal kinetics of induction and the mechanism of enhanced expression were apparent between these early response genes in differentiation-inducer treated H0-1 cells (55). In the case of *c-fos*, IFN- β + MEZ induced an increase in transcription of *c-fos* mRNA that was apparent after 1, 6 and 24 hr, but not after 96 hr treatment (55). In the case of *c-jun*, increased mRNA was apparent after 1, 6, 24 and 96-hour treatment with IFN- β + MEZ. These changes in *c-jun* level did not involve increased transcription, but instead

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resulted from an increase in half-life of the c-jun transcripts (55). In the case of jun-B, IFN- β + MEZ increased both the transcription and steady-state levels of RNA after 1- and 24-hour treatment. High levels of jun-B mRNA were also apparent in H0-1 cells induced to terminally differentiate after treatment with IFN- β + MEZ. The continued increase in c-jun and jun-B mRNA levels in terminally differentiated H0-1 cells suggests that these genes may contribute to maintenance of the terminal differentiation phenotype (55).

D. Autocrine Loops Induced in Human Melanoma Cells Treated with IFN- β plus MEZ.

As indicated above, continuous treatment with IFN- β + MEZ for 96-hour results in terminal differentiation in H0-1 human melanoma cells. This process correlates with specific patterns of gene expression changes, including the induction of two interferon stimulated genes, ISG-15 and ISG-54, and melanocyte growth stimulatory activity (gro/MGSA) (14). These observations suggested the possibility that induction of a reversible commitment to differentiation and terminal differentiation might be associated with the production of differentiation promoting factors (DPFs) (possibly including IFN- β or an IFN- β -like cytokine and melanoma growth stimulatory activity (gro/MGSA)). These DPFs could then induce by an autocrine mechanism the transcription and steady-state mRNA expression of interferon stimulated genes (ISG) and the gro/MGSA gene in H0-1 cells reversibly committed to differentiation or terminally differentiated. To further explore the relationship between treatment time and induction of differentiation and to test the autocrine hypothesis, applicants performed two types of experiments. In the first set of studies (In-Out), H0-1

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cells were treated with various agents (including IFN- β + MEZ, MPA + MEZ, RA + MEZ and MEZ (at a high dose of 50 ng/ml) for 24 hr, cells were washed 2X with DMEM without FBS, DMEM containing 10% FBS was added to cultures and
5 total cytoplasmic RNA was isolated 72 hours later. For the second set of experiments (Conditioned-Medium), cells were processed as indicated above for In-Out experiments except after 72 hour growth in the absence of inducer, medium was collected (and contaminating cells were
10 removed by centrifugation).

Using the In-Out and Conditioned-Medium protocols it was demonstrated that: (a) At the doses employed, IFN- β + MEZ is a more potent inducer of gene expression changes and
15 the only combination capable of inducing terminal differentiation in H0-1 cells; and (b) Conditioned medium from H0-1 cells reversibly committed to differentiate or terminally differentiated by IFN- β + MEZ induce specific programs of gene expression changes in H0-1 cells that
20 are similar to those induced directly by the inducing agents (14). In addition, conditioned medium from IFN- β + MEZ treated H0-1 cells also induces morphologic changes and suppresses growth when added to H0-1 or F0-1 human melanoma cells (data not shown). These results support
25 the hypothesis that induction of terminal differentiation in H0-1 melanoma cells is associated with specific changes in gene expression, some of which may be mediated by or associated with an autocrine feedback mechanism.

30 E. Cloning of Melanoma Differentiation Associated (*mda*) Genes Induced in H0-1 Melanoma Cells treated with IFN- β plus MEZ.

35 The ability to identify and isolate differentially expressed genes between two similar or different cell

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types is now readily achievable using subtraction hybridization (rev. 57, 58). A procedure for constructing subtracted libraries have been developed that is both sensitive and efficient (49). The application of this approach for the identification of genes differentially expressed in H0-1 cells treated with IFN- β plus MEZ is outlined in Figure 8. Tester and driver cDNA libraries are directionally cloned into the commercially available λ Uni-ZAP phage vector. Subtraction hybridization is then performed between double-stranded tester DNA and single-stranded driver DNA prepared by mass excision of the libraries. The subtracted cDNAs are efficiently cloned into the λ Uni-ZAP phage vector that can be easily managed for both screening and gene characterization. The applicability of the procedure was demonstrated by the identification of cDNAs displaying enhanced expression in human melanoma cells, H0-1, induced to terminally differentiate by treatment with IFN- β + MEZ (Fig. 10). A single round of subtraction of untreated H0-1 control (Ind^-) cDNAs from IFN- β + MEZ treated (Ind^+) cDNAs generated a series of cDNAs displaying differential expression in untreated versus differentiation inducer-treated H0-1 cells, termed melanoma differentiation associated (*mda*) cDNAs. Employing the approach briefly described above, a total of 23 differentially expressed *mda* cDNAs have been isolated which represents only a portion of the subtracted H0-1 IFN- β + MEZ cDNA library. Partial sequence analysis of these 23 *mda* genes resulted in the identification of known genes, including a human TPA-inducible gene, the human apoferritin H gene, the IFP-53 (gamma-2 protein) gene, the IL-8 (monocyte-derived chemotactic factor) gene, the vimentin gene, the hnRNP A2 protein gene, human macrophage inflammatory protein

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(GOS19-1) and the IFN- β -inducible gene ISG-56. In addition, 6 cDNAs have been identified which do not have sequences previously reported in any of the gene data bases. As predicted based on the subtraction protocol employed, some of the *mda* genes are induced within 24 hours by: IFN- β and IFN- β + MEZ (e.g., *mda*-1 and *mda*-2); MEZ and IFN- β + MEZ (e.g., *mda*-3); IFN- β , MEZ and IFN- β + MEZ (e.g., *mda*-4); and only by IFN- β + MEZ (e.g., *mda*-5 and *mda*-6) (Fig. 10). A potentially important group of *mda* genes is represented by cDNAs displaying significantly enhanced expression in H0-1 cells treated with IFN- β + MEZ for 96 hours and displaying terminal cell differentiation, i.e., *mda*-5, *mda*-6, *mda*-7 and *mda*-9 (all representing novel genes) (Fig. 15). Additional *mda* cDNAs which may prove of value in understanding growth control in human melanoma cells have been identified which are expressed in both terminally differentiated H0-1 cells and H0-1 cells induced to undergo a reversible suppression in growth by treatment with IFN- β + IFN- γ , i.e., *mda*-4, *mda*-5, *mda*-7 and *mda*-8 (Fig. 15). Increased expression of a number of *mda* genes following treatment with IFN- β + MEZ are not restricted to H0-1 cells, since increased *mda* gene expression is also induced in additional human melanomas induced to terminally differentiate by treatment with IFN- β + MEZ (data not shown). The studies described above indicate the feasibility of using subtraction hybridization to identify genes that may directly mediate or represent markers of terminal differentiation in human melanoma cells.

F. Gene Expression Changes induced in the C8161 Melanoma Cells and Chromosome 6 Microcell C8161 Hybrids.

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Recent studies by Welch et al. (42) indicate that insertion of a normal chromosome 6 (by the microcell chromosome replacement technique) into the C8161 human melanoma cell line results in a suppression of metastatic, but not tumorigenic potential in nude mice. Treatment of C8161 cells for 4 or 7 days with IFN- β + MEZ (1000 units/ml + 10 ng/ml) results in terminal cell differentiation. In contrast, under similar conditions, C8161 cells containing chromosome 6 (Clone 6.1, 6.2 and 6.3) display morphological changes and growth suppression but cells retain proliferative potential, i.e., the combination of agents induces a reversible commitment to differentiation as opposed to terminal differentiation. A lack of terminal differentiation in 6.1, 6.2 and 6.3 cells was demonstrated by removing the test agents and growth in inducer free medium (data not shown). Analysis of gene expression in parental C8161 and 6.1, 6.2 and 6.3 cells indicated differences that correlated with the presence of a normal chromosome 6. Specific differences in gene expression after 4 days incubation with IFN- β and MEZ, alone and in combination, include: (a) induction of IL-8 mRNA (which was identified as an *mda* cDNA in H0-1 cells treated with IFN- β + MEZ) in MEZ and IFN- β + MEZ treated C8161 cells, but not in 6.1, 6.2 or 6.3 cells; (b) induction of HLA Class I antigen mRNA by IFN- β , MEZ and IFN- β + MEZ in C8161, but only by IFN- β and IFN- β + MEZ in 6.1, 6.2 and 6.3 cells; and (c) reduced induction of ISG-15 expression in C8161 cells versus 6.1, 6.2 and 6.3 cells treated with IFN- β and IFN- β + MEZ. The studies briefly described above indicate that IFN- β + MEZ is more effective in inducing terminal differentiation in the less differentiated metastatic C8161 melanoma cells than the more differentiated 6.1, 6.2 and 6.3 cells. This model system should prove useful in determining the role of specific *mda* genes in expression of the

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tumorigenic and metastatic phenotype by human melanoma cells.

DESIGN AND METHODS

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A. Specific Aim #1: Determine the pattern and regulation of expression of the melanoma differentiation associated (*mda*) genes in melanocytes, nevi, radial growth phase melanoma, vertical growth phase melanoma and metastatic melanoma cells.

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1. Rationale and General Approach:

Applicants have tested the hypothesis that human melanoma cells display aberrant patterns of differentiation and by appropriate chemical treatment they can be induced to undergo an irreversible loss in proliferative capacity without a loss of viability, i.e., terminal cell differentiation (10,11,14). Using the combination of IFN- β + MEZ applicants have demonstrated that the reprogramming of human melanoma cells to terminally differentiate can be achieved in vitro (10,11,14). On the basis of a second hypothesis, i.e., terminal differentiation is associated with the selective activation of specific programs of gene expression, applicants have developed and used a modified subtraction hybridization protocol to identify genes displaying enhanced expression under conditions resulting in terminal cell differentiation (49). These studies have resulted in the cloning of a series of genes, termed melanoma differentiation associated (*mda*) genes, which display such specificity. The purpose of the studies to be described below are to: (a) characterize the *mda* genes with respect to their level of regulation in H0-1 melanoma cells, i.e., transcriptional versus

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posttranscriptional mechanisms of induction; (b) determine if the expression of specific *mda* genes correlate with a defined stage in melanoma development; (c) continue screening our subtracted IFN- β + MEZ cDNA library to identify additional *mda* genes which display enhanced expression in growth arrested and terminally differentiated human melanoma cells; and (d) use additional subtraction steps to enrich for genes only expressed at high levels in H0-1 cells induced to terminally differentiate.

(a) Defining the level of regulation of *mda* genes in H0-1 cells treated with IFN- β + MEZ: Initial studies will focus on the mechanism by which IFN- β + MEZ increases the expression of cloned *mda* genes that are significantly upregulated (4- to >20-fold) by this combination of inducing agents in H0-1 cells. The genes to be analyzed will include *mda*-5, *mda*-6, *mda*-7, *mda*-8 and *mda*-9 described in Preliminary Studies, which represent novel IFN- β + MEZ-inducible genes not previously reported in the Gene Bank or the EMBL gene data base. The order of experiments will include: (1) determining the temporal kinetics of induction of the *mda* genes; (2) determining if the level of induction of specific *mda* genes occurs at a transcriptional level; (3) determining if any of the *mda* genes are immediate early (primary) response genes; and (4) determining if differentiation results in an altered stability of the *mda* transcripts.

(i) The screening strategy used to identify the *mda* genes involved Northern hybridization analysis of RNA isolated from H0-1 cells treated with IFN- β , MEZ or IFN- β + MEZ for 24 hr (49). Previous studies indicated that exposure to IFN- β + MEZ for 24 hr resulted in a number of gene expression changes also observed in H0-1 cells induced to

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terminally differentiate after 4 days exposure to this combination of agents (Preliminary Studies) (14). *mda* genes displaying increased expression in H0-1 cells after 24 hr treatment with IFN- β + MEZ were subsequently evaluated for enhanced expression after 4 days treatment with the inducers. *mda*-5, *mda*-6, *mda*-7, *mda*-8 and *mda*-9 genes displayed enhanced expression in H0-1 cells treated with IFN- β + MEZ for 24 or 96 hr. These results indicated that increased expression of the *mda* genes occurred within the first 24 hr of treatment and enhanced expression persisted during terminal cell differentiation. To determine if any of the *mda* genes becomes activated after a short exposure to the inducing agents, temporal kinetic studies will be performed. H0-1 cells will be treated for short-time periods (15, 30, 45, 60 and 120 min) with IFN- β , MEZ and IFN- β + MEZ, cytoplasmic RNA will be isolated, electrophoresed on 0.6% agarose gels, transferred to nylon filters and sequentially hybridized with the various *mda* genes and lastly with GAPDH (as a control for equal RNA levels under the various experimental conditions) (14). RNA from cells treated with the inducers will also be isolated every 2 hr over a 48 hr period to determine if any cell cycle kinetic changes occur in expression of the *mda* genes. An important question that also will be addressed is whether continued expression of any of the *mda* genes is required for maintenance of the terminal differentiation phenotype. This will be determined by analyzing RNA isolated from H0-1 cells treated with IFN- β + MEZ: continuously for 7 days (cells are terminal, but still viable); for 4 days followed by incubation in growth medium without inducers for an additional 10 days (cells remain terminal); and after 10 days in cells treated for 24 hr followed by growth in inducer-free medium (cells regain proliferative potential, i.e., they

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display a reversible commitment to terminal differentiation).

(ii) The studies described above will indicate if any of the *mda* genes is induced early after exposure to IFN- β + MEZ. To determine if IFN- β + MEZ induce expression of any of the *mda* genes by increasing their rates of transcription, nuclear run-on assays will be performed as described previously (55,58,59). Brief Description of Protocol: Nuclei will be isolated from H0-1 cells either untreated (control) or treated for 1, 6 and 24 hr with IFN- β (2000 units/ml), MEZ (10 ng/ml) or IFN- β + MEZ (2000 units/ml + 10 ng/ml). RNA transcripts previously initiated by RNA polymerase II will be allowed to elongate in the presence of [^{3} P]UTP. Nuclear RNA will be isolated, purified by passing through a G-50 sephadex* column followed by denaturing with 0.1M NaOH for 5 min on ice (55). Labeled nuclear RNA will be hybridized to nitrocellulose dot filters containing 2 μ g of plasmid DNA containing the *mda* genes, GAPDH DNA or pBR322 DNA (negative control) which has been denatured by boiling in 0.1M NaOH for 15 min followed by dilution with cold 2M NaCl (55).

(iii) *c-fos*, *c-jun* and *jun-B* are immediate early (primary) response genes, i.e., induction is not dependent on new protein synthesis, but rather utilizes existing transcription factors, in IFN- β + MEZ treated H0-1 cells (55). To determine if any of the *mda* genes is an immediate early (primary) response gene, experiments using the protein synthesis inhibitor cycloheximide will be performed (55). Brief Description of Protocol: Approximately 2×10^6 H0-1 cells will be untreated or treated with IFN- β (2000 units/ml), MEZ (10 ng/ml) or

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IFN- β + MEZ (2000 units/ml + 10 ng/ml) for 1 hr in the absence and presence of 50 μ g/ml cycloheximide (added 15 min prior to any other additions) (55). Total RNA will be isolated, electrophoresed in 0.6 % agarose, transferred to nitrocellulose filters and hybridized sequentially with the *mda* genes and lastly with GAPDH (14, 49, 60). By definition, if any of the *mda* genes are immediate early response genes they will be induced within 1 hour by IFN- β + MEZ in both the absence and presence of cycloheximide. Another indication that the *mda* genes are primary response genes would be the phenomenon of superinduction, i.e., the massive over-accumulation of immediate early response transcripts which occur when cells are treated simultaneously with an inducer and protein synthesis inhibitors (55,61,62).

(iv) The ability of IFN- β + MEZ to increase c-jun expression does not involve an increase in transcription, but rather results from an increase in the stability of c-jun mRNA (55). To determine if IFN- β + MEZ alters the expression of specific *mda* genes by a posttranscriptional mechanism, studies using the transcription inhibitor actinomycin D will be performed (55). Brief Description of Protocol: Approximately 2×10^6 H0-1 cells will be untreated or treated with IFN- β (2000 units/ml), MEZ (10 ng/ml) and IFN- β + MEZ (2000 units/ml + 10 ng/ml) for 24 hr followed by no addition or the addition of actinomycin D (5 μ g/ml) for 30 min, 1 hr, 2 hr and 3 hr prior to RNA isolation (55). The RNAs will be analyzed by Northern hybridization and probing with the different *mda* genes or GAPDH (14,49,55). Radioautograms will be scanned using a densitometer to quantitate cellular RNA levels (55). These studies will indicate if IFN- β + MEZ can alter the stability, i.e., the half-life, of any of the *mda* gene

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transcripts.

Summary: These studies will indicate if *mda-5*, *mda-6*,
mda-7, *mda-8* or *mda-9* are primary response genes and if
5 their enhanced expression in human melanoma cells treated
with IFN- β + MEZ results from a transcriptional and/or a
posttranscriptional mechanism.

(b) Analysis of *mda* gene expression during the process of
10 melanoma development: A basic tenet of our terminal
differentiation hypothesis is that the *mda* genes may
represent genes normally expressed or expressed at higher
levels in melanocytes and/or in the early stages of
melanoma development, i.e., nevi, early radial growth
15 phase (RGP) primary melanoma and/or early vertical growth
phase (VGP) primary melanoma. If this concept is
correct, then a prediction would be that specific *mda*
genes would display reduced expression in late VGP
melanoma and metastatic melanoma versus melanocytes, nevi
20 and early stage melanomas. Support for this hypothesis
comes from preliminary studies indicating that
SV40-transformed human melanocytes express high levels of
mda-5, *mda-6* and *mda-7* mRNA in the absence and in the
presence of IFN- β + MEZ (data not shown). To directly
25 test this hypothesis applicants will analyze RNA obtained
from cell cultures of melanocytes, dysplastic nevi (DN
91D, DN (MM92E)), RGP melanoma (WM 35), early VGP
melanoma (WM 793, WM902b), advanced VGP melanoma (WM
983a, WM 115) and metastatic melanomas (WM 9, MeWo,
30 SK-MEL 28, WM 239) (1,16,21,22,27) (to be supplied by Dr.
Meenhard Herlyn). Applicants realize that cell cultures
may not always reflect processes occurring in vivo,
however, cell cultures will provide an initial indication
of which *mda* genes to emphasize using in situ
35 hybridization approaches with clinical specimens of

melanocytes, pre-malignant skin lesions, primary melanoma and metastatic melanoma. The procedures to be used for in situ hybridization with oligonucleotide probes will be as described by Reed et al. (63) and Biroc et al. (64) (to be performed in collaboration with Dr. Anthony P. Albino, Memorial Sloan-Kettering Cancer Center, New York). In the studies by Reed et al. (63) in situ hybridization with a basic fibroblast growth factor (bFGF) oligonucleotide has been successfully used to determine differential expression of bFGF in melanocytic lineage tissue specimens. As an additional approach for determining *mda* gene expression in clinical specimens, RNA isolated directly from patient samples displaying different stages of melanoma evolution (to be supplied by Dr. Herlyn) will be evaluated by Northern analysis (14,49) and where necessary to increase sensitivity of detection by RT-PCR analysis (65) for expression of the *mda* genes. In previous studies analyzing expression of the epidermal growth factor receptor, a wide spectrum of human central nervous system tumors obtained from patients was evaluated (66). This study clearly indicated that intact and high-quality RNA could be obtained efficiently from patient material and utilized for comparative gene expression studies.

As indicated in Background and Significance, metastatic melanoma cells are often inhibited in their growth by TPA (or MEZ), whereas the in vitro growth of normal melanocytes and nevi are stimulated by TPA (or MEZ) (1,15,16,20-23). Similarly, many metastatic melanoma cell lines are growth inhibited by IFN- β (8,13-15), whereas under optimal growth conditions normal melanocytes are not growth inhibited by IFN- β (even though TPA is incorporated in the growth medium) (15). These results indicate that progression from melanocyte

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to malignant melanoma involves a change in responsiveness to both TPA (or MEZ) and IFN- β . Based on these observations, it would be predicted that a stage-specific effect will be observed in melanocyte lineage cells exposed to the combination of IFN- β + MEZ. If this effect is observed, it will provide a direct test of the potential involvement of the *mda* genes in the process of growth inhibition and terminal differentiation resulting from treatment with IFN- β + MEZ. The melanocyte lineage cell lines, i.e., melanocyte, dysplastic nevus, RGP primary melanoma, early VGP primary melanoma, advanced VGP melanoma and metastatic melanoma (supplied by Dr. Meenhard Herlyn, Wistar Institute, Philadelphia, PA), will be used to directly determine: (1) if the combination of IFN- β + MEZ displays a stage-specific growth inhibitory effect and the induction of terminal differentiation; and (2) if the induction of growth suppression and/or the induction of terminal differentiation in stage-specific melanocyte lineage cells correlates with changes in the expression of specific *mda* genes. These studies will be conducted as described previously (10,14). Brief Description of Protocols: For growth studies: cells will be seeded at 2.5 or 5×10^4 cells/35 mm plate; 24 hr later the medium will be changed with no additions (Control), 1000 and 2000 units/ml of IFN- β , 1, 10 and 50 ng/ml MEZ and combinations of IFN- β + MEZ; cell numbers will be determined daily (with a medium change at day 4) over a seven day period. For reversibility studies, cells will be treated for 24 hr or 4 days with the different inducers followed by growth in inducer free medium for an additional 7 to 14 days at which time cell numbers will be determined. RNA will be isolated from cells treated for 24 hr, 4 days and 7 days and analyzed by Northern

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blotting hybridization for expression of the *mda* genes as described previously (14). Biochemical markers for growth suppression and differentiation will include: an analysis of P2P levels using appropriate monoclonal antibodies and Western blotting analysis (Preliminary Studies) (52,53); antigenic markers, such as the GD3 ganglioside and Class II MHC, and fluorescence activated cell sorter analysis as described previously (50,67,68); and a determination of melanin levels as described previously (69).

Studies designed to identify stage-specific effects of IFN- β + MEZ and the role of the *mda* genes in growth suppression and terminal cell differentiation in melanocyte lineage cells will be aided by using three recently described model systems. These will include: (A) the transformed human melanocyte cell lines 10Wras/early and 10Wras/late (supplied by Dr. Anthony P. Albino, Memorial-Sloan Kettering Cancer Center, NY, NY) (20); (B) the metastatic human melanoma cell line C8161 and the tumorigenic but non-metastatic C8161 clones containing a normal human chromosome 6 (supplied by Dr. Dan Welch, Milton S. Hershey Medical Center, Hershey, PA) (42,43); and (C) RGP or early VGP primary human melanomas (WM 35, WM 1341B and WM 793) which have been selected by injection with matrigel in nude mice for a more progressed tumorigenic and metastatic phenotype (e.g., 35-P1-N1, 35-P1-N2, 35-P1-N3, 1341-P1-N1, 1341-P1-N2, 1341-P2-N1 etc. (cell line: passage number; nude mouse number)) (supplied by Dr. Robert S. Kerbel, Sunnybrook Medical Center, Toronto, Canada) (25,70). The 10Wras/early and 10Wras/late cells are human melanocytes transformed by a retrovirus containing the viral Ha-ras oncogene which display specific traits associated with melanoma progression (20). 10Wras/early cells display

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TPA dependence, are nontumorigenic in nude mice, and express many of the antigenic markers present in normal melanocytes (20). In contrast, the 10Wras/late cells are inhibited by TPA, tumorigenic in nude mice, contain many of the cytogenetic changes seen in metastatic melanoma (including modifications in chromosome 1, 6 and 9) and express many of the same growth factor genes as metastatic human melanoma (20). As discussed in Preliminary Studies, applicants have begun to analyze gene expression changes in IFN- β + MEZ treated C8161 cells and C8161 cells containing a microcell-transferred normal chromosome 6 (6.1, 6.2 and 6.3). Unlike C8161 cells, 6.1, 6.2 and 6.3 cells are not metastatic in nude mice (42) and they are not induced to terminally differentiate when treated with the same concentration of IFN- β + MEZ resulting in terminal differentiation in C8161 cells. 6.1, 6.2 and 6.3, therefore, may represent human melanoma cells which have been reverted to a less advanced stage in melanoma development. As discussed previously, since surgical removal of RGP or early stage VGP primary human melanomas results in a cure of this disease, it has not previously been possible to analyze more aggressive variants derived from the same early stage melanomas. Dr. Kerbel and colleagues (25) have potentially overcome this problem by injecting RGP and early VGP primary human melanomas in combination with matrigel into nude mice. Tumors which then developed were found to be tumorigenic in nude mice without the requirement for matrigel and they also acquired a "cytokine resistance phenotype" which is associated with melanoma progression (25,70). These three cell systems should prove extremely valuable in determining if *mda* gene expression correlates with specific stages of melanoma progression. To test this possibility applicants will conduct similar experiments as described

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previously (4. A. 1. a) using these stage-specific cell lines. If our hypothesis suggesting that the response of melanocyte lineage cells to IFN- β + MEZ is stage-specific is correct, then applicants would predict that the effect of these agents on growth, *mda* gene expression and terminal cell differentiation would be greater in 10Wras/late vs. 10Wras/early cells, C8161 cells vs. chromosome 6 containing C8161 clones and the more progressed vs. the less progressed RGP and early VGP primary melanoma cell lines.

Summary: These studies will indicate if a direct relationship exists between the state of progression of melanoma cells and *mda* gene expression. They will also indicate if the response to IFN- β + MEZ induced growth suppression, *mda* gene expression and terminal cell differentiation is directly related to melanoma progression.

(c) and (d) Identification of additional *mda* genes which display enhanced expression in growth arrested and/or terminally differentiated human melanoma cells: As indicated in Preliminary Studies (E), only a small percentage (approximately 2.5%) of our subtracted IFN- β + MEZ H0-1 library has been screened for differentially expressed genes associated with growth suppression and terminal differentiation in H0-1 cells. It is therefore conceivable that a number of additional *mda* genes, which still remain to be identified, are present in the subtracted IFN- β + MEZ H0-1 library. The initial subtraction hybridization approach applicants have used has resulted in the identification of cDNAs displaying enhanced expression in H0-1 cells treated with: IFN- β and IFN- β + MEZ; MEZ and IFN- β + MEZ; IFN- β , MEZ and IFN- β + MEZ; and only IFN- β + MEZ (49). To identify additional

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mda genes which are preferentially expressed at elevated levels in cells induced to terminally differentiate following treatment with IFN- β + MEZ applicants will perform additional subtraction hybridization steps.

5 Brief Description of Protocol: cDNA libraries will be constructed from H0-1 cells treated with IFN- β (2000 units/ml) or MEZ (10 ng/ml) for 2, 4, 8, 12 and 24 hr as previously described (49,57). The IFN- β and MEZ cDNA libraries will be converted into single-stranded DNA

10 which will then be biotinylated using photoactivatable biotin and used as the Driver DNA as described previously (49). The H0-1 IFN- β + MEZ (*Ind*⁺) subtracted cDNA library will be converted into double stranded DNA and the double-stranded inserts will be isolated and used as

15 the Tester DNA (49). The Driver DNA will then be subtracted away from the Tester DNA resulting in an enriched H0-1 IFN- β + MEZ (*Enriched-Ind*⁺) subtracted cDNA library (49). As an alternate approach to specifically

20 identify genes displaying increased elevation in terminally differentiated melanoma cells, cDNA libraries will be constructed from H0-1 cells treated for 4 or 7 days with IFN- β , MEZ and IFN- β + MEZ. Subtraction hybridization will then be conducted as described (49) using these libraries to identify cDNAs only expressed at

25 increased levels in IFN- β + MEZ-treated terminally differentiated H0-1 cells.

Summary: Additional screening of our current H0-1 IFN- β + MEZ (*Ind*⁺) offers the potential of identifying more

30 differentially expressed mda genes. By constructing additional cDNA libraries from H0-1 cells treated singularly with IFN- β or MEZ and subtracting this information away from cDNA libraries prepared from H0-1 cells treated with IFN- β + MEZ, the identification of

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additional *mda* genes displaying enhanced expression specifically in terminally differentiated melanoma cells should result.

- 5 B. Specific Aim #2: Analyze the relationship between
mda gene expression and the induction of reversible
commitment to differentiation, growth suppression without
the induction of differentiation, DNA damage and stress
responses and induction of terminal differentiation in
10 human melanoma and other model differentiation systems.

1. Rationale and General Approach:

Induction of terminal differentiation in human melanoma
15 cells, as well as other cell types such as myoblasts,
neuroblastoma and leukemic cells, is associated with an
irreversible loss in proliferative ability (rev 13,71).
It is therefore reasonable to assume that some of the *mda*
genes applicants have identified may also display
20 enhanced expression in growth arrested melanoma (and
other cell types) or melanoma cells (and other cell
types) treated with various DNA damaging and
chemotherapeutic agents which also induce growth-related
changes. Indeed, preliminary studies indicate that
25 *mda*-4, *mda*-5 and *mda*-8 exhibit increased expression in
terminally differentiated H0-1 cells as well as H0-1
cells induced to undergo reversible growth suppression by
treatment with IFN- β + IFN- γ (50). In addition, *mda*-4
also displays increased expression in H0-1 cells
30 reversibly growth suppressed by caffeic acid phenethyl
ester (72), vinblastine, tumor necrosis factor- α and
X-irradiation. Additional *mda* genes have been identified
which display enhanced expression only in H0-1 and other
metastatic human melanoma cells treated with IFN- β + MEZ
35 (i.e., they appear to be melanoma specific) or in H0-1

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and dissimilar cell types induced to lose proliferative capacity, including human breast and colon carcinoma (i.e., they appear to be growth and or differentiation specific and not melanoma specific). Based on these preliminary observations, it appears that specific *mda* genes may be restricted to melanoma lineage cells induced to lose growth potential and become terminally differentiated, while other *mda* genes may represent key genes involved in growth control processes in diverse cell types. It will, therefore, be important to determine whether changes in the expression of specific *mda* cDNAs are restricted to human melanoma cells induced to terminally differentiate or whether they also display modified expression during other programs of terminal differentiation, DNA damage and growth arrest. The studies described below are designed to determine: (a) the spectrum of cellular changes which induce enhanced *mda* gene expression in human melanoma and other cell types; and (b) if induction of growth suppression and terminal differentiation in other cell types results in the enhanced expression of specific *mda* genes.

(a) Analysis of *mda* gene expression in human melanoma (and other cell types) treated with growth suppressing agents, DNA damaging agents and chemotherapeutic agents: Since terminal differentiation in H0-1 cells is associated with an irreversible loss in proliferative capacity (10,11,14), the *mda* genes applicants have identified may represent genes involved in both cell growth and terminal cell differentiation. To explore the relationship between cell growth and terminal differentiation, studies will be conducted to determine the types of agents and treatment protocols which can induce *mda* gene expression in human melanoma and other human cell types. The agents and treatments to be tested

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will include: growth suppression (incubation in reduced serum levels), heat shock, gamma irradiation, ultraviolet irradiation (UVA and UVB), carcinogenic and mutagenic agents (methyl methanesulfonate, ethyl methanesulfonate and 4-nitroquinoline-oxide), demethylating agents (5-azacytidine, phenyl butyrate), chemotherapeutic agents (vinblastine, vincristine, adriamycin, cis-platinum), tumor necrosis factor- α , protein synthesis inhibitors (cycloheximide, puromycin, anisomycin), DNA synthesis inhibitors (amphidicolin, hydroxylurea, ara-C), transcription inhibitors (actinomycin D), topoisomerase inhibitors (camptothecin), poly-ADP-ribose inhibitors (3-aminobenzamide), protein kinase C activators (TPA, teleocidin, synthetic PKC activators (ADMB and DHI) (73-75)) and phosphatase inhibitors (calyculin, okadaic acid). Initial studies will focus on H0-1 cells. Subsequent investigations will involve other melanoma cells (representing different stages of melanoma progression) and additional human cell types (normal fibroblast and epithelial cells, neuroblastoma, glioblastoma, carcinomas (prostate, breast and colon) and sarcomas). Brief Description of Protocol: H0-1 cells (or the other experimental cell type employed) will be treated with the various agents for different time periods (ranging from 1 hr to 24 hr; or with certain treatment protocols for 4 and 7 days) and with different doses of the test treatment or agent. RNA will be isolated, electrophoresed in 0.6 % agarose gels, transferred to nylon filters and hybridized sequentially with the different *mda* genes and lastly with GAPDH. If specific pathways appear to induce an *mda* gene then further biochemical studies will be conducted. For example, if PKC activators induce specific *mda* genes then studies will be performed using specific inactive analogs and inhibitors of PKC to determine the relationship

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between PKC activation and induction of gene expression. Similarly, if a treatment protocol or agent is found to induce or enhance expression of an *mda* gene, then subsequent studies will be conducted to determine if this change in gene expression is transcriptional or post-transcriptional (see Specific Aim #1 for experimental details).

Summary: The studies briefly outlined above will indicate if *mda* gene expression can be induced in H0-1, other human melanoma cells and additional human cell types, by treatment with agents which can alter growth and/or differentiation. They will provide initial information relative to potential biochemical pathways which may mediate the induction or enhanced expression of the *mda* genes. These experiments will also identify which *mda* gene(s) to use in studies (described in C. Specific Aim #3.) designed to determine the potential functional significance of *mda* gene expression changes in the control of growth and differentiation in human melanoma cells and other human cell types.

(b) Analysis of *mda* gene expression during the process of terminal differentiation in human promyelocytic leukemia (HL-60) and human skeletal muscle cultures: Specific *mda* genes are expressed in human melanoma and additional human cell types undergoing growth suppression with and without the induction of terminal differentiation. To explore this phenomenon further and to determine if any of the *mda* genes are also expressed at elevated levels in additional differentiation model systems applicants will conduct experiments using the HL-60 promyelocytic leukemic cell line (76) and human skeletal muscle cells (77,78). TPA induces macrophage differentiation in HL-60 cells (79), whereas dimethyl

5 sulfoxide (DMSO) results in granulocytic differentiation in HL-60 cells (80). In addition, growth of HL-60 cells in medium containing DMSO for 5 days followed by growth in TPA results in cells which switch from a granulocytic to monocytic differentiation program (81). These studies indicate that specific monocytic or granulocytic lineages can be induced in HL-60 cells by appropriate chemical manipulation. By growing HL-60 cells in incremental increases of TPA and DMSO, applicants have isolated
10 variant populations which display a quantitative resistance to either TPA- or DMSO-induced differentiation (76). These resistant variants do not, however, display cross-resistance to other inducers, i.e., TPA induces a similar pattern of differentiation in parental HL-60 and HL-60/DMSO^R cells and DMSO induces a similar pattern of differentiation in parental HL-60 and HL-60/TPA^R cells. Although IFN- α A and IFN- β induce growth suppression in parental HL-60, TPA-resistant HL-60 (HL-60/TPA^R) and DMSO-resistant HL-60 (HL-60/DMSO^R) cells, they do not
15 induce these cells to differentiate terminally (76). However, the combination of IFN- α A or IFN- β and TPA results in a synergistic growth suppression and the induction of terminal differentiation in both parental and HL-60/TPA^R cells (76). Similarly, the combination of
20 IFN- α A or IFN- β and DMSO results in synergistic growth suppression and the induction of terminal differentiation in both parental and HL-60/DMSO^R cells (76). This experimental model will prove extremely valuable in determining if a correlation exists between enhanced *mda* gene expression and either growth suppression or growth
25 suppression with the induction of specific programs of terminal differentiation in human myeloblastic leukemic cells. Brief Description of Protocols: Parental HL-60, HL-60/TPA^R and HL-60/DMSO^R cells will be incubated with
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IFN- β (2000 units/ml) $\pm$ inducer (TPA at 10^{-9} and 10^{-6} M or DMSO 0.9 to 1.5%) for 1, 3 and 7 days (with fresh medium $\pm$ additions added at day 4). Cell numbers and terminal differentiation (as monitored by the presence of morphologically mature cells and the ability of cells to reduce nitroblue tetrazolium (NBT) (granulocyte specific) or the production of nonspecific esterase (macrophage specific) will be determined (84). RNA will be isolated, electrophoresed in 0.6% agarose gels, transferred to nylon filters and hybridized sequentially with the different *mda* genes and lastly with GAPDH (14, 49). Since the induction of both growth suppression and terminal differentiation is concentration- and compound-dependent in parental HL-60 and variant HL-60 cells, the studies outlined above will indicate if a relationship exists between the degree of growth suppression and/or the induction of terminal differentiation and expression of the different *mda* genes in HL-60 cells.

Methods are available for the in vitro growth of myogenic muscle satellite cells obtained from normal adult human skeletal muscle (82). These cultures recapitulate normal myogenesis, a process that can be followed with specific morphologic and biochemical markers and thus provides a useful system for assessing the effects of various agents on the process of cellular differentiation in human cells (77, 78, 82). Using this model system, applicants have previously demonstrated that TPA (and related compounds) inhibit spontaneous and induced myogenesis, whereas IFN- α A enhances myogenesis (77, 78). Inhibition or enhancement of differentiation in human skeletal muscle cultures is associated with either the suppression or induction, respectively, of specific morphologic changes

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(development of multinucleated myotubes) and changes in creatine kinase isoenzyme transition from CK-BB to the CK-MM form (77,78). Applicants have also developed SV40-immortalized human skeletal muscle cells which fail to undergo terminal differentiation when treated with IFN- α A (83). This experimental model will prove extremely valuable in determining if any of the *mda* genes are differentially expressed during the induction of terminal differentiation or growth suppression in human skeletal muscle cells. Brief Description of Protocols: Muscle cultures will be grown from human skeletal muscle biopsy specimens obtained from diagnostic evaluation (from patients of either sex and ranging from 6 months to 50 years of age) as previously described (77,78,82). Prior to myoblast fusion, the cells will be trypsinized and plated at 2×10^6 cells/10 cm tissue culture plate, cultures will be incubated with IFN- β (2000 units/ml) and MEZ (10 ng/ml), alone and in combination, for 1, 4 and 7 days (with an appropriate medium change at day 4). Cell numbers will be determined and differentiation will be monitored using morphologic (myoblast fusion) and biochemical (creatine kinase isoenzyme transition from CK-BB to CK-MM) criteria (77,78,82). RNA will be isolated, electrophoresed in 0.6 % agarose gels, transferred to nylon filters and hybridized sequentially with the different *mda* genes and lastly with GAPDH. When only small quantities of RNA are available, RT-PCR (65) will be used to determine expression of the appropriate *mda* gene in early passage human skeletal myoblast cultures. In this model system, IFN- β promotes skeletal muscle differentiation whereas MEZ inhibits differentiation. By employing specific concentrations of IFN- β + MEZ, it is possible to obtain either an enhancement in differentiation, no change in

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differentiation or an inhibition in differentiation (78).
This system should permit a direct determination of the
relationship between *mda* gene expression and the
induction of myogenesis in human skeletal muscle
5 cultures.

Summary: The studies described briefly above will
indicate if any of applicants *mda* genes display altered
expression during the induction of growth suppression and
10 terminal differentiation in human myeloid leukemic cells
and human skeletal muscle cells. If changes are
observed, subsequent studies could be conducted to
determine if the *mda* genes display enhanced expression in
other differentiation models, including the U937 human
15 monoblastic leukemia cell which can be induced to undergo
macrophage differentiation (81), the PC12 rat
pheochromocytoma cell line which can be induced to
undergo neuronal differentiation (85) and human
neuroblastoma cells which can be induced to differentiate
20 terminally when treated with retinoic acid or other
agents. In addition, if *mda* gene expression changes are
observed in HL-60 cells induced to terminally
differentiate, further studies would be conducted using
HL-60 cells and other inducing agents which result in
25 growth arrest without differentiation, DNA damage and
apoptosis and terminal cell differentiation (with and
without apoptosis). These studies would indicate which
cellular and biochemical changes in HL-60 cells result in
induction of specific programs of *mda* gene expression.

30

C. Specific Aim #3: Isolate full-length cDNAs of *mda*
genes that may be involved in melanoma differentiation or
progression and directly determine their potential
35 functional role in the differentiation and progression of

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human melanoma.

1. Rationale and General Approach:

5 The ability to analyze the functional significance of specific *mda* genes will require the isolation of full-length cDNAs. Once a full-length cDNA has been identified for a specific *mda* gene it can be used to: (1) produce its encoded protein using an in vitro translation
10 system; (2) generate polyclonal antibodies specific for peptide regions of the encoded protein; (3) determine the location of the *mda* gene product in human melanoma cells and in tissue sections from patients; (4) determine the effect of overexpression of the *mda* gene on induction of
15 growth suppression and terminal differentiation; and (5) determine the effect of blocking *mda* gene expression (using antisense oligomers or expression vector constructs) on the ability of IFN- β + MEZ to induce growth suppression and terminal cell differentiation.
20 The approaches to be utilized to identify full-length *mda* cDNAs, in vitro translate the full-length *mda* cDNAs, produce antibodies against specific peptides of the encoded proteins, determine the location of the encoded proteins and to construct and analyze the effect of sense
25 and antisense oligomers and expression vector constructs on growth and differentiation in H0-1 and other cell types is described below.

(a) Strategy for isolating full-length *mda* cDNAs: Rapid
30 amplification of cDNA ends (RACE) is a procedure for amplification of nucleic acid sequences from a mRNA template between a defined internal site and an unknown sequence representing either the 3' or 5' end of the mRNA (86-88). The RACE procedure will be used to obtain the
35 complete sequence of the full-length *mda* cDNAs (5' ends)

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using the sequences (already determined) as the templates. Two types of gene-specific primers will be synthesized: the RT primer for reverse transcription and the AMP primer for PCR amplification. The sequence of the AMP primer is located upstream of the RT primer. First strand cDNA synthesis is initiated from the RT primer. After first strand cDNA synthesis, the original mRNA template is destroyed with RNase H and unincorporated dNTPs and RT primers are separated from cDNA using Centricon spin filters (Amicon Corp.). An oligo-DA anchor sequence is then added to the 3' end of the cDNA using TdT (terminal d transferase) and dATP. PCR amplification is accomplished using the AMP primer and a mixture of oligo(dT)-adapter primer/adapter primer (1:9). The adapter primer contains a specific sequence which, in the form of dsDNA, can be recognized and digested by the restriction enzymes SalI and XhoI. Following amplification, the RACE products will be digested with specific restriction enzymes (which do not cut the cDNA internally) and cloned into an appropriate plasmid (such as pBlueScript). The clones with specific inserts will be selected by screening (using DNA filter hybridization) and multiple independent clones of each gene will be used simultaneously for DNA sequencing to eliminate possible errors in sequence determination as a result of misincorporations occurring during the PCR amplification process.

(b) Characterization of full-length *mda* cDNAs: Full-length *mda* cDNAs will be used to obtain information about the *mda*-encoded proteins and the potential function of these genetic elements in regulating growth and differentiation of human melanoma cells. As will be described below, in vitro translation will be used to obtain the *mda*-encoded proteins. Once the protein

structure is determined, synthetic peptides will be constructed and used to generate antibodies specific for defined regions of the *mda* proteins. Antibodies will be used to determine the location of the *mda* proteins in melanoma and other cell types and tissue sections.

(i) Determination of protein structure and development of polyclonal antibodies specific for *mda* genes: Once full-length *mda* cDNAs have been isolated and sequenced, information will be available relative to the presence of open reading frames and the amino acid composition of the putative proteins encoded by these *mda* genes. To directly determine the size of the proteins encoded by the *mda* genes, full-length cDNAs will be subcloned into the pGEM-1 vector and transcribed in vitro using SP6 polymerase (Promega Corp., Madison, WI) as described (89). The in vitro transcribed RNA will be translated in a rabbit reticulocyte lysate (Amersham) in the presence of [³⁵S]methionine according to the manufacturer's instructions and dialyzed against 10 mM Hepes, pH 7.9, 1 mM MgCl₂, 1 mM dithiothreitol, 20% glycerol, 100 mM NaCl, 100 μM ZnCl₂ at 4°C overnight. Protein products will be analyzed by electrophoresis in an SDS/10-20% polyacrylamide gradient gel. Based on predicted amino acid structure of the protein, i.e., hydrophobicity value, antigenicity value and turn structure based on the deduced sequences of the *mda* cDNA, specific amino acids will be chosen for generating synthetic peptides (75,90). Synthetic peptides will then be used to generate polyclonal antibodies (Hazelton Laboratories, Denver, PA or Cappel Laboratories, Durham, NC). Brief Description of Protocol: The synthetic peptides will be conjugated with carrier proteins, bovine serum albumin and CGG (chicken γ-globulin) as described (90). Two and one-half

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- mg of peptide and 5 mg of carrier protein in double-distilled water will be incubated with 20 mg of either ethyl CDI (1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride) (Sigma) or morpho CDI (1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluenesulfonate) (Sigma) in water at room temperature for 2 hours and dialyzed against PBS, pH 7.2. Rabbits will be immunized with 0.5 mg of BSA peptide (conjugated by ethyl CDI) emulsified with complete Freund's adjuvant at 2-week intervals. The antisera against peptides will then be purified by affinity chromatography coupled with CGG peptide (conjugated by morpho CDI), excluding the by-product generated in the conjugation reaction. The polyclonal antibodies will be titrated (by 1:1 serial dilution, beginning with a 1:50 dilution) on human melanoma cells, either untreated or treated with IFN- β + MEZ, by ELISA assays as described previously (91).
- (ii) Immunostaining of cultured cells and sectioned patient samples with anti-mda peptide antibodies: Based on preliminary studies using SV40-immortalized human melanocytes, applicants would predict that specific mda gene products would be produced at increased levels in normal melanocytes versus melanoma cells. To test this possibility and to determine if differences are apparent in specific stages of melanoma development or as a consequence of IFN- β + MEZ treatment, melanocytes, dysplastic nevi and different staged melanomas (RGP, early VGP, late VGP and metastatic) will be cultured on coverslips. Twenty four hr later, one group of cultures will receive a media change without additions and the other will receive fresh medium with 2000 units/ml of IFN- β + 10 ng/ml of MEZ. After an additional 24 hr, cultures will be washed 3X with PBS followed by fixation

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with 3.7% formalin for 30 min (90,91). The slides will then be washed 3X with PBS and treated with 0.2% Triton^{*} X-100/PBS for 5 min at room temperature. After extensive washing with PBS and blocking nonspecific binding sites with 2% egg albumin/PBS, the cells will be incubated with affinity purified anti-*mda* peptide antibody or preimmunized control sera for 30 min. The slides will then be incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin antibody for 30 min followed by examination with a fluorescence microscope after extensive washing with 0.1% SDS/PBS (90). Studies will also be conducted to determine if the anti-*mda* peptide antibodies react with melanocyte/melanoma lineage tissue. Reactivity of the anti-*mda* peptide antibodies toward sectioned clinical samples representing normal melanocytes, dysplastic nevi, and RGP, early VGP, late VGP and metastatic melanoma (supplied by Drs. Albino and Herlyn) (63) will be analyzed as previously described (90,91).

(iii) Analysis of the effect of forced *mda* expression on growth and differentiation in human melanoma cells: A key question will be whether expression of any of the *mda* genes can directly inhibit melanoma growth or induce morphological, biochemical or gene expression changes associated with growth suppression and the induction of differentiation without the addition of inducer (IFN- β + MEZ). To determine the effect of overexpression of specific *mda* genes on growth and differentiation in human melanoma cells applicants will employ expression vectors containing full-length *mda* cDNAs. Full-length *mda* cDNAs will be cloned into an expression vector containing an inducible promoter, such as the dexamethasone (DEX)-inducible MMTV promoter, and a selectable antibiotic resistance gene, such as the neomycin

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resistance gene, e.g., pMAMneo construct (Clonetech). Transfer of this *mda-S* construct into human melanoma cells will permit the direct isolation of cells containing the specific *mda-S* gene and will permit regulation (by altering DEX concentrations) of the level of expression of the specific *mda* gene. This approach may be necessary, since continuous increased expression of specific *mda* genes under control of promoters such as the cytomegalovirus (CMV) or the β -actin promoter may result in loss of proliferative ability and/or terminal differentiation in human melanoma cells. By using different MMTV promoter-driven constructs containing different *mda* genes and different antibiotic resistance genes, it will also be possible to construct melanoma cells containing several *mda-S* inducible gene constructs. An additional advantage of a regulatable expression vector system will be the stable nature of the cell clones and the ability to determine if transient or prolonged *mda-S* expression is required to induce an irreversible loss of proliferative capacity and terminal differentiation in H0-1 cells. Brief Description of Protocols: H0-1 cells (additional human melanoma cell lines or other human tumor cell lines) will be seeded at 2×10^6 cells/100 mm plate, 24 hr later cells will be transfected by electroporation with the *mda-S* construct (alone if a selectable gene is present in the construct or in conjunction with a cloned selectable gene) as described (92). Antibiotic resistant colonies will be selected and isolated as pure clones (92, 93). The presence of the inserted gene will be determined by Southern blotting and expression of the endogenous and exogenous gene will be distinguished by RNase protection assays (58,94). The ability of DEX to enhance expression of the *mda-S* gene in appropriate H0-1 cell lines will be

determined by growing cells in the presence or absence of the appropriate inducer (10^{-9} to 10^{-5} M DEX or 2000 units/ml of IFN- β) for 24 hr prior to isolating and characterizing RNA expression (95). Under conditions of non-induction
5 (absence of DEX) or induction (presence of DEX) cells containing the MMTV-inducible *mda-S* constructs will be evaluated for alterations in growth (10, 14), increases in melanin synthesis (10, 69), modification in cell surface antigenic phenotype (67, 68, 72), changes in the
10 levels of P2Ps (52, 53), patterns of gene expression (14) and the induction of irreversible loss of proliferation (terminal differentiation) (10,11,14) using previously described protocols.

15 Potential Outcome of *mda-S* Expression Construct Studies:
The studies briefly described above could result in one of three potential outcomes. First, a single *mda-S* construct could induce growth suppression, gene
expression changes associated with differentiation and
20 terminal cell differentiation in the absence of IFN- β + MEZ. This would provide compelling evidence that this specific *mda* gene is a controlling element in regulating growth and differentiation in human melanoma cells.
Second, specific *mda-S* constructs could modify only a
25 portion of the changes induced by IFN- β + MEZ in human melanoma cells, i.e., induce growth suppression, induce growth suppression and some markers of differentiation or induce only some of the gene expression changes associated with differentiation without affecting growth.
30 If this occurs, then it may be possible by using a combination of *mda-S* constructs, which induce different components of the differentiation program in human melanoma cells, to induce a loss of proliferative capacity and terminal differentiation in human melanoma

cells. Third, *mda-S* constructs could display no effects on growth or differentiation programs in H0-1 cells. This result would of course be the least informative outcome. It would suggest that the specific *mda-S* constructs tested are not controlling elements in melanoma differentiation, although these genes may be altered during the induction of growth suppression and terminal differentiation in human melanoma cells by IFN- β + MEZ.

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(iv) Analysis of the effect of antisense oligomers and expression vector constructs on growth and differentiation in human melanoma cells: Antisense RNA is an effective approach for interfering with the expression of specific target genes (96). The antisense transcript has a sequence complementary to the target mRNA and can presumably anneal to the mRNA and disrupt normal processing or translation (96). Mechanism(s) by which antisense constructs inhibit gene functions include: a direct interference in translation by binding to the ribosomal assembly (translation initiation) site and/or coding regions; stimulation of mRNA degradation by RNase H which specifically cleaves double-stranded RNA hybrids; and blocking translocation of the mRNA from the nucleus into the cytoplasm (96). Previous studies have demonstrated that antisense constructs or oligodeoxyribonucleotides (oligomers) of specific genes, such as *c-myc* and *Egr-1*, can modulate cell growth and/or differentiation (84,97-101). To determine the effect of blocking *mda* gene expression on growth and differentiation in human melanoma cells applicants will conduct experiments using antisense oligomers and expression vectors containing antisense constructs.

35 In the first set of experiments applicants will determine

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if oligomers complementary to specific regions of the *mda* genes can induce growth suppression and/or changes in the expression of genes previously shown to be altered in H0-1 cells treated with IFN- β + MEZ, i.e., c-myc, IL-8, FIB, MGSA/gro, ISG-15, c-jun and jun-B (14,55). As indicated above, antisense oligomers have been employed successfully to alter cell physiology in a number of cell lines and they have been shown to affect the expression of many genes (rev. 102,103). *mda*-gene specific oligomers complementary to the translation initiation sites or 5'-coding regions will be synthesized with phosphorothioate modification (phosphorothioate oligodeoxynucleotide) to increase nuclease resistance (96,102,103). Although unmodified oligomers have been utilized in many laboratories, they can be degraded rapidly by nucleases present in serum-supplemented medium (96,102). Since experiments with H0-1 and the other cell types will utilize serum-containing medium, applicants will use phosphorothioate oligodeoxynucleotides as opposed to unmodified oligomers.

Brief Description of Protocols: For growth studies, H0-1 cells (or other test cell lines) will be seeded at 2.5×10^4 cells/35mm tissue culture plate and 24 hr later fresh medium with IFN- β + MEZ (2000 units/ml + 10 ng/ml), various concentrations (1 to 200 μ M) of the 3',5'-phosphorothioate end-capped *mda* antisense oligomer complementary to several target sequences of the 5' region of the *mda* gene (20 bases in length) (*mda*-AS oligomer) or the combination of IFN- β + MEZ and the *mda*-AS oligomer will be added. As appropriate controls, cultures will also receive similar concentrations of a 5',3'-phosphorothioate end-capped *mda* sense oligomer (*mda*-S oligomer) or the *mda*-S oligomer plus IFN- β + MEZ (65, 84, 104). Cell numbers will be determined daily over a 7 day period (with medium changes

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with the appropriate additions every 48 hr) to identify the correct *mda*-AS oligomer to use and the concentration of *mda*-AS oligomer required to block the effect of IFN- β + MEZ on growth inhibition in H0-1 cells. If an appropriate *mda*-AS oligomer is identified it will be used for subsequent studies to determine the effect of this *mda*-AS oligomer on gene expression (14) and biochemical (10, 52, 53, 69) and immunological changes (67, 68, 72) induced in H0-1 cells treated with IFN- β + MEZ. For gene expression studies, H0-1 cells (or other test cell lines) will be seeded at 2.5×10^6 cells/100 mm tissue culture plate and the appropriate concentration of *mda*-AS or *mda*-S oligomer will be added 24 hr later. RNA will be isolated after an additional 24 hr or 96 hr (with a medium change after 48 hr), electrophoresed in 0.6 % agarose gels, transferred to nylon filters and hybridized sequentially with *c-myc*, *c-jun*, *jun-B*, ISG-15, MGSA/*gro*, IL-8, FIB and lastly with GAPDH (14). Assays will also be conducted to determine if *mda*-AS oligomers can prevent or diminish the biochemical, immunological and/or cellular changes induced in H0-1 cells by the combination of IFN- β + MEZ. Parameters to be monitored, by previously described techniques, include morphology (10), melanin synthesis (10,69), antigenic expression (67,68,72) and levels of P2Ps (52,53). Possible Outcome of Studies: The experiments described above could produce one of three possible outcomes. First, specific *mda*-AS oligomers could inhibit the ability of IFN- β + MEZ to induce growth suppression, gene expression changes associated with differentiation and terminal cell differentiation. Second, specific *mda*-AS oligomers could modify only a portion of the changes induced by IFN- β + MEZ in H0-1 cells, i.e., reverse growth suppression, reverse both growth suppression and terminal

differentiation or reverse only some of the gene expression changes. Third, specific *mda*-AS oligomers could display no effect on the growth suppression or the differentiation program induced by IFN- β + MEZ.

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A positive result using *mda*-AS oligomers in blocking specific cellular and biochemical changes in H0-1 cells treated with IFN- β + MEZ would provide strong evidence for a relationship between expression of a specific *mda*
10 gene and a defined component of the differentiation process. However, a negative effect of a specific *mda*-AS gene could occur for many reasons including lack of stability of the antisense oligomer, inadequate quantity of the antisense oligomer or the requirement for the
15 expression of multiple *mda* genes in the differentiation process. To further explore the effect of *mda*-AS gene expression in inhibiting chemical induction of differentiation in human melanoma cells experiments will also be conducted using *mda*-AS cDNAs (*mda* cDNAs cloned in
20 an antisense orientation) in expression vector constructs. Since one question applicants intend to address is the relationship between levels of expression and tissue specific expression of specific *mda*-AS cDNAs and cellular phenotype, applicants will use several
25 expression vector constructs under the transcriptional control of different promoters. The constructs to be used should result in high level targeted, constitutive or inducible transcriptional control of the *mda*-AS cDNAs. The same *mda*-AS cDNA will be cloned into an expression
30 vector containing a promoter which will permit: high levels of constitutive expression (β -actin promoter (84,94)); enhanced expression after interferon treatment (interferon responsive sequence (IRS) promoter (pTKO-1) (105); inducible expression in the presence of
35 dexamethasone- (MMTV) inducible promoter (93, 106); or

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expression in melanocyte/melanoma lineage cells (tyrosinase promoter (107)). In addition, by using different selectable genes, i.e., neomycin, histidinol, hygromycin etc., either present in the expression vector
5 construct or by cotransfection (93) it will be possible to construct human melanoma cells which contain several *mda*-AS cDNAs. In summary, the use of different promoters will permit a direct evaluation of constitutive, inducible and cell-lineage specific (targeted) expression
10 of the *mda*-AS cDNA on growth and differentiation in human melanoma and other cell types. If expression of a specific *mda*-AS cDNA inhibits the ability of IFN- β + MEZ to induce growth suppression, changes in gene expression and terminal differentiation in H0-1 and other human
15 melanoma cells, this would provide strong evidence for a direct relationship between expression of this *mda* cDNA and the growth and differentiation process in human melanoma cells. Brief Description of Protocols: H0-1 cells (additional human melanoma cell lines or other
20 human tumor cell lines) will be seeded at 2×10^6 cells/100 mm plate, 24 hours later cells will be transfected by electroporation with the *mda*-AS construct (alone if a selectable gene is present in the construct or in conjunction with a cloned selectable gene) as
25 described (92, 93). Antibiotic resistant colonies will be selected and isolated as pure clones (92, 93). The presence of the inserted gene will be determined by Southern blotting and expression of the endogenous and exogenous gene will be distinguished by RNase protection
30 assays (58, 94). The ability of interferon or dexamethasone to enhance antisense in a pTKO-1 (interferon inducible promoter) or a pMAMneo (DEX inducible) construct, respectively, will be determined by growing cells in the presence or absence of the

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appropriate inducer (10^{-9} to 10^{-5} M DEX or 2000 units/ml of IFN- β) for 24 hours prior to isolating and characterizing RNA expression (95, 105). Similar cellular (growth and morphology), biochemical (melanin and P2P levels), immunological (antigenic expression) and molecular (gene expression) parameters as used to study *mda*-S constructs will be used to study *mda*-AS constructs.

Summary: These studies will provide important information about the *mda* encoded gene products and they will indicate if perturbations in the expression of specific *mda* genes can directly modify growth or the differentiation process in human melanoma cells.

D. Specific Aim #4: Isolate and characterize the promoter region of *mda* genes and analyze their regulation in human melanocytes, nevi and melanoma.

1. Rationale and General Approach:

In order to elucidate the mechanism underlying the transcriptional regulation of the *mda* genes it will be necessary to analyze the promoter regions of these genes. This will be important for studies aimed at determining regulatory control of the *mda* genes including chemical induction, autoregulation, tissue specific regulation and developmental regulation. Once the appropriate promoters of the *mda* genes have been isolated, studies can be conducted to identify relevant trans-regulatory factors (nuclear proteins) which bind to specific cis-regulatory elements and activate or repress the expression of the *mda* genes. The experiments outlined below are designed to: [a] clone the promoter region of specific *mda* genes and analyze their function in untreated and IFN- β + MEZ

treated melanoma cells; [b] identify cis-regulatory elements in the promoter region of specific *mda* genes which are responsible for IFN- β + MEZ induction in human melanoma cells; and [c] identify and characterize
5 trans-regulatory elements which activate (or repress) expression of the *mda* genes.

(a) Cloning the promoter region of the *mda* genes and testing their function in untreated and IFN- β + MEZ
10 treated human melanoma cells: To isolate the promoter region of the *mda* gene a human genomic library will be constructed by partial digestion of H0-1 human melanoma genomic DNA with the restriction enzyme Sau3AI and then
15 ligation into dephosphorylated vectors (phage or cosmid vectors) (92). Using the *mda* cDNA gene to screen the library, clones will be identified which contain both the *mda* gene and its 5' and 3' regions (92). Since the insert in a phage or cosmid vector is too large to
20 analyze (i.e., 10 to 30 Kb) and the structure and size of the genomic DNA for the *mda* genes are not known, the inserts will be subcloned (to an approximate size of 2 Kb) in order to identify the potential promoter region (92). Two types of probes will be used for subcloning:
25 one containing the coding region of the *mda* gene and the other a synthetic oligonucleotide (a 20 mer) complementary to the sequence located in the 5' non-translated region of the *mda* gene. The genomic DNA containing the *mda* gene in phage or cosmid vector will be
30 digested with a series of restriction enzymes, electrophoresed on 0.8% agarose gels and transferred to nylon filters (92,108). This Southern blot will then be probed with the coding region of the *mda* gene and the synthetic oligonucleotide. This double probing method
35 will permit a more effective identification of the promoter region than utilizing a single probe. Putative

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promoter inserts of approximately 2 Kb in size will be subcloned into various CAT expression vector constructs (including pSV0CAT, pUVOCAT or pChlorAce) for later functional analysis (92,108,109).

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The putative promoter region of the *mda* genes will be sequenced by the Sanger dideoxynucleotide procedure (110). The transcription start site (+1) will be determined by primer extension as described previously (108, 111). Dried total RNA samples of H0-1 melanoma cells with or without treatment with IFN- β + MEZ will be resuspended in 20 μ l of 10 mM PIPES (pH 6.4)-400 mM NaCl containing 5'-end ^{32} P-labeled oligonucleotides (a 25 mer, complementary to the 5'-untranslated region of the *mda* gene) made by the T4 nucleotide kinase method. After 3 hr incubation at 60°C, 80 μ l of 50 mM Tris-HCl (pH8.2)-5 mM MgCl₂-10 mM dithiothreitol-5 mM deoxyguanosine nucleoside triphosphates-25 μ l of dactinomycin per ml containing 10 U of avian myeloblastosis virus reverse transcriptase will be added and the primer extension reaction will be allowed to proceed for 1 hr at 42°C. Following phenol-chloroform extraction and ethanol precipitation, samples will be electrophoresed on 6% acrylamide-8 M urea sequencing gels (108,111). From the length of the extended products, the transcription initiation site of the *mda* gene can be determined.

To functionally analyze the various *mda* promoters, appropriate *pmdaCAT* constructs will be transfected into melanoma cells by the CaPO₄ method or electroporation (Gene Pulser*, Bio-Rad) as previously described (93, 108, 109). Cell extracts will be prepared 48 hr after transfection by washing cells 3X with PBS, pelleting manually resuspended cells and lysing cells by three

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cycles of freeze-thawing. The CAT reaction will then be performed by adding 55 μ l of cell extracts into a reaction mixture consisting of 5 μ l of ^{14} C-chloramphenicol, 70 μ l of 1 M Tris-HCl (pH8.0) and 20 μ l of 4 mM butyryl CoA (108,109). After incubation at 37°C for 2 hr, the reaction mixture will be extracted with ethyl acetate or xylene and CAT activity will be determined either by scintillation counting or by TLC (108,109). If CAT expression can be detected in specific pmdaCAT construct transfected human melanoma cells after treatment with IFN- β + MEZ, but not in untreated cultures, this would indicate that the promoter region of the specific mda gene contains appropriate cis-acting elements responsive to induction by IFN- β + MEZ. If no induction is apparent, further subcloning and screening of cosmid or phage clones would be performed until an mda promoter of sufficient length to mediate CAT induction in differentiation inducer-treated human melanoma cells is obtained. A potentially useful series of CAT constructs have been developed by United States Biochemical (Cleveland, Ohio), referred to as the pChlorAce series. The basic plasmid pChlorAce-B does not contain a eucaryotic promoter or enhancer sequences and is therefore dependent on incorporation of a functional promoter upstream from the CAT gene for expression of CAT activity. The construct, pChlorAce-E, contains an enhancer sequence permitting a direct test of a functional promoter CAT-junction for testing mda promoter sequences. The promoter containing plasmid, pChlorAce-P, incorporates an SV40 promoter upstream from the CAT gene allowing the insertion of enhancer elements in both orientations upstream or downstream from the promoter-CAT transcriptional unit. It will be possible by inserting different parts of the mda gene into the pChlorAce-P

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construct to directly identify the enhancer component of the *mda* gene. The control plasmid, pChlorAce-C contains both the promoter and enhancer and can function as an internal standard for comparing promoter and enhancer strengths.

An important question will be whether specific *mda* genes display tissue- and developmental-specific expression. Once specific *mda* promoters are identified they can be used to address this issue. *mda*-CAT constructs will be tested for levels of expression in untreated and IFN- β + MEZ-treated normal human melanocytes, dysplastic nevi and RGP, early VGP, late VGP and metastatic melanomas. Experiments will also be performed to determine if the *mda* promoters can function in either untreated or IFN- β + MEZ-treated non-melanocyte/melanoma lineage cells. Positive expression in specific target cells would suggest that the appropriate regulatory proteins are either constitutively present or inducible in these cells.

(b) Identifying cis-elements in the *mda* promoter responsible for induction by IFN- β + MEZ in human melanoma cells: Once a functional *mda* promoter has been identified studies will be conducted to locate cis-elements responsible for induction of expression by IFN- β + MEZ. The approach to be used will involve the construction and evaluation for CAT inducible activity of a series of 5'-deletion mutants, 3'-deletion mutants, internal-deletion mutants and linker-scanning mutants of the *mda* promoter regions. The details for constructing the 5'-deletion, 3'-deletion and internal-deletion mutants and screening for CAT activity has been described previously (108,109). Linker-scanning mutants will be constructed by combining 5'- and 3'-deleted *mda* promoter

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sequences after filling gap regions with a linker DNA sequence (such as an EcoRI linker) (92). The structure of the various mutants will be determined by sequence analysis (95,110). Since the promoter region for the *mda* gene is located in front of the CAT reporter gene in the various *pmdaCAT* constructs, the CAT activity for each construct can be measured using liquid scintillation and/or TLC (108, 109). This will permit a direct comparison of CAT transcriptional activity of the mutant promoter to that of the unmodified *mda* promoter. These studies will result in the identification of specific cis-regulatory elements responsible for IFN- β + MEZ induction of enhanced *mda* gene expression in human melanoma cells.

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(c) Identifying trans-acting nuclear proteins induced by IFN- β + MEZ which mediate transcriptional enhancing activity of *mda* genes in human melanoma cells: The current view on regulation of eucaryotic gene expression suggests that trans-acting proteins bind to specific sites within cis-elements of a promoter region resulting in transcriptional activation (rev in 112,113). Studies employing various mutant *mda* promoter CAT constructs will provide information relative to cis-regulatory elements mediating activity of the *mda* promoters. Experiments will be performed to identify trans-acting factors (nuclear proteins) and determine where these factors interact with cis-regulatory elements. To achieve this goal, two types of studies will be performed, one involving DNase-I footprinting (methylation interference) assays (108,109) and the second involving gel retardation (gel shift) assays (109,114).

For DNase-I footprinting assays nuclear extracts from human melanoma cells, untreated or treated with IFN- β +

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MEZ, will be prepared as described previously (109,113,115). DNase-I footprinting assays will be performed as described (108,109). The cis-element (approximately 200 bp) for IFN- β + MEZ induction, identified from the experiments described above, will be terminally labeled with ^{32}P and incubated with crude nuclear extracts from untreated or IFN- β + MEZ treated human melanoma cells using the protocols described previously (108,109). The reaction mixture which has been digested with DNase-I enzyme will be terminated and the digested products will be analyzed on an 8% sequencing gel (108,109). The differential protection between nuclear extracts from induced and uninduced human melanoma cells will provide relevant information concerning the involvement of trans-acting factors in activation and the location of specific sequences in the cis-regulatory elements of the *mda* promoter mediating this activation. If differential protection is not detected using this approach, the sensitivity of the procedure can be improved by using different sized DNA fragments from the *mda* promoter region or by using partially purified nuclear extracts (109).

As a second approach for investigating the interaction between cis-acting elements in the *mda* promoter and trans-acting factors in mediating transcriptional control, gel shift assays will be performed as described previously (109,114). For this assay, ^{32}P -labeled cis-elements will be incubated with nuclear extracts from untreated or IFN- β + MEZ treated human melanoma cells and reaction mixtures will be resolved on 5 or 8% polyacrylamide gels (109,114). After autoradiography, the pattern of retarded DNAs on the gel will provide information concerning the interaction between

trans-acting factors and specific regions of the
cis-elements in the *mda* promoters. Non-labeled
cis-elements (self-competition) will be added as a
competitor to duplicate samples to eliminate the
possibility of non-specific binding and to confirm that
the interaction is really conferred by the trans-acting
factor. To begin to identify the trans-acting factors,
different non-labeled DNAs (for example TATA, CAT, TRE,
Sp-I binding site, NFkB, CREB, TRE, TBP, etc.) can be
used as competitors in the gel shift assay to determine
the relationship between the trans-acting factors and
other previously identified transcriptional regulators.

Summary: These studies will result in the identification
and cloning of the *mda* promoter region, the
identification of cis-regulatory elements in the *mda*
promoters and the identification of trans-regulatory
elements which activate (or repress) expression of *mda*
genes.

F. Future Studies: The currently proposed research will
result in the characterization of specific genes which
may be involved in or mediate growth control, response to
chemotherapeutic and DNA damaging agents and terminal
differentiation in human melanoma cells. Once
appropriate *mda* genes are identified they can
subsequently be used to directly test their functional
role in development and melanocyte/melanoma biology.
Experiments can also be performed to define the role of
these genes in non-melanoma target cells and additional
programs of differentiation. Future studies which also
are not within the scope of the present proposal would
include: (a) evaluation of the effect of specific *mda*-S
and *mda*-AS constructs on the growth (tumorigenic and
metastatic potential) and differentiation of human

melanoma (and other tumor cell types) grown in vivo in nude mice; (b) generation of transgenic mice displaying both tissue and non-tissue specific overexpression of individual or combinations of *mda* genes to evaluate the effect of modified *mda* expression on development; and (c) homologous recombination-mediated gene targeting techniques to produce mice with specific *mda* null mutations to evaluate the effect of lack of expression of specific *mda* genes on tissue and embryo development.

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References of the Third Series of Experiments

1. Herlyn, M., Human melanoma: development and progression. Cancer Metastasis Rev. 9:101-112, 1990.
- 5 2. Kerbel, R.S., Growth dominance of the metastatic cancer cell: cellular and molecular aspects. Adv. Cancer. Res. 55:87-132, 1990.
- 10 3. Clark, W., Tumor progression and the nature of cancer. Br. J. Cancer 64:631-644, 1991.
- 15 4. Clark, W.H., Jr, The Skin. In: Rubin, E & Farber, JL, Eds, Pathology, Lippincott Press Inc., New York, pp. 1194-1259, 1988.
- 20 5. Sachs L., Control of normal cell differentiation and the phenotypic reversion of malignancy in myeloid cells. Nature 274: 535-539, 1978.
6. Jimenez, J.J. & Yunis, A.A., Tumor cell rejection through terminal cell differentiation. Science 238: 1278-1280, 1987.
- 25 7. Waxman S., Rossi, G.B. & Takaku, F. (eds.), The Status of Differentiation Therapy of Cancer, vol I, Serono Symposia Pubs, 45:1-422, Raven Press, NY, 1988.
- 30 8. Fisher, P.B. & Rowley, P.T., Regulation of growth, differentiation, and antigen expression in human tumor cells by recombinant cytokines: applications for the differentiation therapy of human cancer. In: The Status of Differentiation Therapy of Cancer, vol II, Waxman S., Rossi, G.B. & Takaku, F. (eds.), 35

Serono Symposia Pubs, Raven Press, NY, 82:201-214, 1991.

- 5 9. Waxman, S., Rossi, G.B. & Takaku, F. (eds.), In: The Status of Differentiation Therapy of Cancer, vol II, Serono Symposia Pubs, Raven Press, NY, 85:1-451, 1991.
- 10 10. Fisher, P.B., Prignoli, D.R., Hermo, H. Jr., Weinstein, I.B. & Pestka, S., Effects of combined treatment with interferon and mezerein on melanogenesis and growth in human melanoma cells. J. Interferon Res. 5:11-22, 1985.
- 15 11. Fisher, P.B., Hermo H, Jr., Solowey, W.E., Dietrich, M.C., Edwalds, G.M., Weinstein, I.B., Langer, J.A., Pestka, S., Giacomini, P., Kusama, M. & Ferrone, S., Effect of recombinant human fibroblast interferon and mezerein on growth, differentiation, immune
20 interferon binding and tumor associated antigen expression in human melanoma cells. Anticancer Res. 6:765-774, 1986.
- 25 12. Ahmed, M.A., Guarini, L., Ferrone, S. & Fisher, P.B., Induction of differentiation in human melanoma cells by the combination of different classes of interferons or interferon plus mezerein. Ann. NY Acad. Sci. 567:328-333, 1989.
- 30 13. Ahmed, M.A., Nielsch, U., Guarini, L., Hermo, H. Jr. & Fisher, P.B., Modulation of differentiation: a potential mechanism by which interferons induce antitumor activity. In: Mechanisms of
35 Differentiation: Modulation of Differentiation by Exogenous Agents, Fisher, P.B. (ed.), vol II, pp

2174354

-190-

1-56, CRC Press Inc, Boca Raton, Florida, 1990.

- 5 14. Jiang, H., Su, Z.Z., Boyd, J. & Fisher, P.B., Gene expression changes induced in human melanoma cells undergoing reversible growth suppression and terminal cell differentiation. Mol. Cell Differentiation 1:41-66, 1993.
- 10 15. Krasagakis, K., Garbe, C., Kruger, S. & Orfanos, E., Effects of interferons on cultured human melanocytes in vitro: interferon-beta but not -alpha or -gamma inhibit proliferation and all interferons significantly modulate the cell phenotype. J. Invest. Dermatol. 97:364-372, 1991.
- 15 16. Herlyn, M., Mancianti, M.L., Janbrosic, J., Bolen, J.B. & Koprowski, H., Regulatory factors that determine growth and phenotype of normal human melanocytes. Exp. Cell Res. 179:322-331, 1988.
- 20 17. Mufson, R.A., Fischer, S.M., Verma, A.K., Gleason, G.L., Slaga, T.J. & Boutwell, R.K., Effects of 12-O-tetradecanoylphorbol-13-acetate and mezerein on epidermal ornithine decarboxylase activity, isoproterenol-stimulated levels of cyclic adenosine 3':5'-monophosphate, and induction of mouse skin tumors in vivo. Cancer Res. 39:4791-4796, 1979.
- 25 18. Slaga, T.J., Fischer, S.M., Nelson, K. & Gleason, G.L., Studies on the mechanism of skin tumor promotion: evidence for several stages in promotion. Proc. Natl. Acad. Sci. USA, 77:3659-3663, 1980.
- 30 19. Huberman, E., Heckman, C. & Langenbach, R.,
- 35

-191-

Stimulation of differentiated functions in human melanoma cells by tumor-promoting agents and dimethyl sulfoxide. Cancer Res. 39:2618-2624, 1979.

5

20. Albino, A.P., Sozzi, G., Nanus, D.M., Jhanwar, S.C. & Houghton, A.N., Malignant transformation of human melanocytes: induction of a complete melanoma phenotype and genotype. Oncogene 7:2315-2321, 1992.

10

21. Herlyn, M., Kath, R., Williams, N., Valyi-Nagy, I. & Rodeck, M., Growth-regulatory factors for normal, premalignant and malignant human cell in vitro. Adv. Cancer Res. 54:213-234, 1989.

15

22. Rodeck, U., Herlyn, M., Menssen, H.D., Furlanetto, R.W. & Koprowski, H., Metastatic but not primary melanoma cell lines grow in vitro independently of exogenous growth factors. Int. J. Cancer 40:687-690, 1987.

20

23. Halaban, R., Kwon, B.S., Ghosh, S., Bovis, P.D. & Baird A., bFGF as an autocrine growth factor for human melanomas. Oncogene Res 3:177-186, 1988.

25

24. Cornil, I., Theodorescu, D., Man, S., Herlyn, M., Jambrosic, J. & Kerbel, R.S., Fibroblast cell interactions with human melanoma cells affect tumor cell growth as a function of tumor progression. Proc. Natl. Acad. Sci. USA 88:6028-6032, 1991.

30

25. Kobayashi, H., Man, S., MacDougall, J.R., Graham, C.H., Lu, C. & Kerbel, R.S., Variant sublines of early-stage human melanomas selected for tumorigenicity in nude mice express a multicytokine-resistant phenotype. Amer. J. Pathol., 144: 776-786, 1994.

35

-192-

26. Albino, A.P., The role of oncogenes and growth
5 factors in progressive melanoma- genesis. Pigment
Cell Res. Suppl. 2:199-218, 1992.
27. Rodeck, U., Melber, K., Kath, R.E., Menssen, H-D,
Varello, M., Atkinson, B. & Herlyn, M., Constitutive
10 expression of multiple growth factor genes by
melanoma cells but not normal melanocytes. J
Invest. Dermatology 97:20-26, 1991.
28. Yamanishi, D.T., Buckmeier, J.A. & Meyskens, F.L.,
15 Jr, Expression of c-jun, jun-B, and c-fos
proto-oncogenes in human primary melanocytes and
metastatic melanomas. J. Invest. Dermatology
97:349-353, 1991.
29. Chenevix-Trench, G., Martin, N.G. & Ellem, K.A.O.,
20 Gene expression in melanoma cell lines and cultured
melanocytesL correlation between levels of c-src-1,
c-myc and p53. Oncogene 5: 1187-1193, 1990.
30. Albino, A.P., Davis BM & Nanus DM, Induction of
25 growth factor RNA expression in human malignant
melanoma: markers of transformation. Cancer Res.
51:4815-4820, 1991.
31. Limon, J., Dal Cin, P., Sait, S.N.J., Karakousis, C.
30 & Sandberg, A.A., Chromosome changes in metastatic
human melanoma. Cancer Genetics Cytogenetics
30:201-211, 1988.
32. Parmiter, A.H., Balaban, G., Herlyn, M., Clark, W.H.
35

-193-

Jr. & Nowell, P.C., A t(1;19) chromosome translocation in three cases of human malignant melanoma. Cancer Res. 46:1526-1529, 1988.

- 5 33. Pedersen, M.I. & Wang, N., Chromosomal evolution in the progression and metastasis of human malignant melanoma: a multiple lesion study. Cancer Genetics Cytogenetics 41:185-201, 1989.
- 10 34. Dracopoli, N.C., Alhadeff, B., Houghton, A.N. & Old, L.J., Loss of heterozygosity at autosomal and X-linked loci during tumor progression in a patient with melanoma. Cancer Res. 47:3995-4000, 1987.
- 15 35. Cowan, J.M., Halaban, R. & Francke, U., Cytogenetic analysis of melanocytes from premalignant nevi and melanomas. J. Natl. Cancer Inst. 80:1159-1164, 1988.
- 20 36. Dracopoli, N.C., Harnett, P., Bale, S.J., Stanger, B.Z., Tucker, M.A., Housman, D.E. & Kefford, R.F., Loss of alleles from the distal short arm of chromosome 1 occurs late in melanoma tumor progression. Proc. Natl. Acad. Sci. USA
25 86:4614-4618, 1989.
- 30 37. Trent, J.M., Stanbridge, E.J., McBride, H.L., Meese, E.U., Casey, G., Araujo, D.E., Witkowski, C.M. & Nagle R,B., Tumorigenicity in human melanoma cell lines controlled by introduction of human chromosome 6. Science 247:568-571 (1990).
38. Trent, J.M., Cytogenetics of human malignant melanoma. Cancer Metastasis Rev, 10:103-113, 1991.

-194-

39. Cannon-Albright, L.A., Goldgar, D.E., Meyer, L.J.,
Lewis, C.M., Anderson, D.E., Fountain, J.W., Hegi,
M.E., Wiseman, R.W., Petty, E.M., Bale, A.E.,
Olopade, O.I., Diaz, M.O., Kwiatkowski, D.J.,
5 Piepkorn, W., Zone, J.J. & Skolnick, M.H.,
Assignment of a locus for familial melanoma, MLM, to
chromosome 9p13-p22. Science 258:1148-1152, 1992.
40. Fountain, J.W., Karayiorgou, M., Ernstoff, M.S.,
10 Kirkwood, J.M., Vlock, D.R., Titus-Ernstoff, L.,
Bouchard, B., Vijayasaradhi, S., Houghton, A.N.,
Lahti, J., Kidd, V.J., Housman, D.E. & Dracopoli,
N.C. Homozygous deletions within human chromosome
band 9p21 in melanoma. Proc. Natl. Acad. Sci. USA
15 89:10557-10561, 1992.
41. Fountain, J.W., Bale, S.J., Housman, D.E. &
Dracopoli, N.C., Genetics of melanoma. Cancer
Surveys 9:645-671, 1990.
20
42. Welch, D.R., Chen, P., Miele, M.E., McGary, C.T.,
Bower, J.M., Stanbridge, E.J. & Weissman, C.,
Microcell-mediated transfer of chromosome 6 into
metastatic human C8161 melanoma cells suppresses
25 metastasis but does not inhibit tumorigenicity.
Oncogene, 9: 255-562, 1994.
- 30
44. Henco, K., Brosius, J., Fujisawa, A., Fujisawa,
35 J-I., Haynes, J.R., Hochstadt, J., Koviacic, T.,

-195-

Pasek, M., Schambock, A., Schmid, J., Todokoro, K., Walchli, M., Nagata, S. & Weissman, C., Structural relationship of human interferon alpha genes and pseudogenes. J. Mol. Biol. 185:227-260, 1985.

5

45. Diaz, M.O., Ziemien, S., LeBeau, M.M., Pitha, P., Smith, C.D., Chilcote, R.R. & Rowley, J.D.; Homozygous deletion of the α - and β_1 -interferon genes in human leukemia and derived cell lines. Proc. Natl. Acad. Sci. USA 85:5259-5263, 1988.

10

46. Diaz, M.O., Rubin, C.M., Harden, A., Ziemien, S., Larson, R.A., LeBeau, M.M. & Rowley, J.D., Deletions of interferon genes in acute lymphocytic leukemia. New England J. Med. 322:77-82, 1990.

15

47. Miyakoshi, J., Dobler, K.D., Allalunis-Turner, J., McKean, J.D.S., Petruk, K., Allen, P.B.R., Aronyk, K.N., Weir, B., Huyser-Wierenga, D., Fultun, D., Urtasun, R.C. & Day, R.S. III, Absence of IFNA and IFNB genes from human malignant glioma cell lines and lack of correlation with cellular sensitivity to interferons. Cancer Res. 50:278-283, 1990.

20

25

30

49. Jiang, H.H. & Fisher, P.B., Use of a sensitive and efficient subtraction hybridization protocol for the identification of genes differentially regulated during the induction of differentiation in human

-196-

melanoma cells. Mol. Cell Different. 1: 285-299, 1993.

50. Graham, G.M., Guarini, L., Moulton, T.A., Datta, S.,
5 Ferrone, S., Giacomini, P., Kerbel, R.S. & Fisher,
P.B., Potentiation of growth suppression and
modulation of the antigenic phenotype in human
melanoma cells by the combination of recombinant
human fibroblast and immune interferon. Cancer
10 Immunol. Immunother. 32:382-390, 1991.
51. Kiguchi, K., Collart, F.R., Henning-Chubb, C. &
Huberman, E., Induction of differentiation in
melanoma cells by inhibitors of IMP dehydrogenase:
15 altered patterns of IMP dehydrogenase expression and
activity. Cell Growth & Differ. 1:259-270, 1990.
52. Minoo, P., Sullivan W., Solomon, L.R., Martin, T.E.,
Toft, D.O. & Scott, R.E., Loss of proliferative
20 potential during terminal differentiation coincides
with decreased abundance of a subset of
heterogeneous ribonuclear proteins. J. Cell Biol.
109:1937-1946, 1989.
- 25 53. Witte, M.M. & Scott, R.E., Repression of two
proliferation proteins during senescence and
terminal differentiation in normal and SV40
transfected human keratinocytes: P2Ps and SC40 large
T-antigen. Mol. Cell Differ, 2:185-195, 1993.
- 30 54. Melber, K., Zhu, G. & Diamond, L., SV40-transformed
human melanocyte sensitivity growth inhibition by
t h e p h o r b o l e s t e r
12-O-tetradecanoylphorbol-13-acetate. Cancer Res.
35 49:3650-3655, 1989.

55. Jiang, H., Waxman, S. & Fisher, P.B., Regulation of c-fos, c-jun, and jun-B gene expression in human melanoma cells induced to terminally differentiate. Mol Cell Differ. 1(2):197-214, 1993.
- 5
56. Schweinfest, C.W. & Papas, T.S., Subtraction hybridization: an approach to the isolation of genes differentially expressed in cancer and other biological systems (Review). Int. J. Cancer 1:499-506, 1992.
- 10
57. Reddy, P.G., Su, Z.Z. & Fisher, P.B., Identification and cloning of genes involved in progression of transformed phenotype. In: Methods in Molecular Genetics, vol 1, Adolph, K.W. (ed.), Academic Press, pp 68-102, Orlando, Fl, 1993.
- 15
58. Duigou, G.J., Babiss, L.E., Iman, D.S., Shay, J.W. & Fisher, P.B., Suppression of the progression phenotype in somatic cell hybrids occurs in the absence of altered type 5 adenovirus gene expression. Mol. Cell. Biol. 10:2027-2034, 1990.
- 20
59. Su, Z.Z., Austin, V.A., Zimmer, S.G. & Fisher, P.B., Defining the critical gene expression changes associated with expression and suppression of the tumorigenic and metastatic phenotype in Ha-ras-transformed cloned rat embryo fibroblasts. Oncogene, 8:1211-1219, 1993.
- 25
60. Jiang, H., Su, Z.Z., Datta, S., Guarini, L., Waxman, S. & Fisher, P.B., Fludarabine phosphate selectively inhibits growth and modifies the antigenic phenotype of human glioblastoma multiforme cells expressing a multidrug resistance phenotype. Int. J. Oncol
- 30
- 35

-198-

1:227-239, 1992.

- 5 61. Lau, L.F. & Nathans, D., Expression of a set of growth-related immediate early genes in BALB/c3T3 cells: coordinate regulation with c-fos and c-myc. Proc. Natl. Acad. Sci. USA 84:1182-1186, 1986.
- 10 62. Kouzarides, T. & Ziff, E., The role of the leucine zipper in fos-jun interaction. Nature 336:646-651, 1988.
- 15 63. Reed, J.A., McNutt, N.S. & Albino, A.P., Differential expression of basic fibroblast growth factor (bFGF) in melanocytic lesions demonstrated by in situ hybridization: implications for tumor progression. Amer. J. Pathology 144: 329-336, 1994.
- 20 64. Biroc, S.L., Murphy-Erdosh, C., Fisher, J.M. & Payan, D.G., The use of ³²P-labeled oligonucleotides for in situ hybridization of vertebrate embryo frozen sections. BioTechnique 15:250-254, 1993.
- 25 65. Abdollahi, A., Lord, K.A., Hoffman-Liebermann, B. & Liebermann, D.A., Interferon regulatory factor 1 is a myeloid differentiation primary response gene induced by interleukin 6 and leukemia inhibitory factor: role in growth inhibition. Cell Growth & Different. 2:401-407, 1991.
- 30 66. Bruce, J.N., Duigou, G.J. & Fisher, P.B., Expression of the epidermal growth factor receptor in human central nervous system tumors. In: Molecular Diagnostics of Human Cancer, Furth, M. & Greaves, M.

(Eds), Cancer Cell, vol 7, pp 363-370, Cold Spring Harbor Press Inc, NY, 1989.

- 5 67. Guarini, L., Temponi, M., Edwalds, G.M., Vita, J.R.,
Fisher, P.B. & Ferrone, S., In vitro differentiation
and antigenic changes in human melanoma cell lines.
Cancer Immunol Immunother 30:363-370, 1989.
- 10 68. Guarini, L., Graham, G.M., Jiang, H., Ferrone, S.,
Zucker, S. & Fisher, P.B., Modulation of the
antigenic phenotype of human melanoma cells by
differentiation-inducing and growth-suppressing
agents. Pigment Cell Res. Suppl. 2:123-131, 1992.
- 15 69. Fisher, P.B., Babiss, L.E. & Miranda, A.F.,
Measurement of the effect of interferons on cellular
differentiation in murine and human melanoma
cultures. In: Methods Enzymol, Interferons, Pestka,
S. (ed.), 119:611-618, Academic Press, Orlando, Fla,
20 1986.
- 25 70. MacDougall, J.R., Kobayashi, H. & Kerbel, R.S.,
Responsiveness of normal/dysplastic melanocytes and
melanoma cells from different lesional stages of
disease progression to the growth inhibitory effects
of TGF- β . Mol. Cell Differentiation 1:21-40, 1993.
- 30 71. Fisher, P.B. & Grant, S., Effects of interferon on
differentiation in normal and tumor cells.
Pharmacol. Therapeut. 27:143-166, 1985.
- 35 72. Guarini, L., Su, Z.z., Zucker, S., Lin, J.,
Grunberger, D. & Fisher, P.B., Inhibition of growth
and modulation of antigenic phenotype in human
melanoma and glioblastoma multiforme cells by

-200-

caffeic acid phenethyl ester (CAPE). Cell Mol. Biol. 38:513-527, 1992.

73. Wender, P.A., Konrad, F.K., Sharkey, N.A.,
5 Dell'Aquila. M.L. & Blumberg, P.C., Analysis of the
phorbol ester pharmacore on protein kinase C as a
guide to the rational design of new classes of
analogs. Proc. Natl. Acad. Sci. USA. 83:4214-4219,
1986.
- 10 74. Leon, J.A., Gutierrez, M.C., Jiang, H., Estabrook,
A., Waxman, S. & Fisher, P.B., Modulation of the
antigenic phenotype of human breast carcinoma cells
by modifiers of protein kinase C activity and
15 recombinant human interferons. Cancer Immunol.
Immunother. 35:315-324, 1992.
- 20 75. Leon, J.A., Goldstein, N.I. & Fisher, P.B., New
approaches for the development and application of
monoclonal antibodies for the diagnosis and therapy
of human cancer. Pharmacol. Therap. 61: 237-278,
1994.
- 25 76. Grant, S., Bhalla, K., Weinstein, I.B., Pestka, S.,
Mileno, M.D. & Fisher, P.B., Recombinant human
interferon sensitizes resistant myeloid leukemic
cells to induction of terminal differentiation.
Biochem. Biophys. Res. Commun. 130:379-388, 1985.
- 30 77. Fisher, P.B., Miranda, A.F., Mufson, R.A.,
Weinstein, L.S., Fujiki, H., Sugimura, T. &
Weinstein, I.B. Effects of teleocidin and the
phorbol ester tumor promoters on cell
transformation, differentiation and phospholipid
35 metabolism. Cancer Res. 42:2829-2835, 1982.

- 5 78. Fisher, P.B., Miranda, A.F., Babiss, L.E., Pestka, S. & Weinstein, I.B. Opposing effects of interferon produced in bacteria and of tumor promoters on myogenesis in human myoblast cultures. Proc. Natl. Acad. Sci. USA 80:2961-2965, 1983
- 10 79. Rovera, G., Santoli, D. & Damansky, C. Human promyelocytic leukemia cells in culture differentiate into macrophage-like cells when treated with phorbol diester. Proc. Natl. Acad. Sci. USA 76: 2779-2803, 1979.
- 15 80. Collins, S.J., Ruscetti, F.W., Ruscetti, R.E., Gallagher, R. & Gallo, R.C. Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds. Proc. Natl. Acad. Sci. USA 75:2458-2462, 1978.
- 20 81. Liebermann, D., Hoffman-Liebermann, B. & Sachs, L. Regulation of gene expression by tumor promoters. II. Control of cell shape and developmental programs for macrophages and granulocytes in human myeloid leukemic cells. Int. J. Cancer 28:285-291, 1981.
- 25 82. Miranda, A.F., DiMauro, S. & Somer, H. Isoenzymes as markers of differentiation. In: Muscle Regeneration, DiMauro, S. (Ed.), pp 453-473; Raven Press, NY, 1979.
- 30 83. Miranda, A.F., Babiss, L.E. & Fisher, P.B. Transformation of human skeletal muscle cells by simian virus 40 (SV40). Proc. Natl. Acad. Sci. USA 80:6581-6585, 1983.

2174354

-202-

- 5 84. Nguyen, H.Q., Hoffman-Liebermann, B. & Liebermann, D.A. The zinc finger transcription factor Egr-1 is essential for and restricts differentiation along the macrophage lineage. Cell 72:197-209, 1993.
- 10 85. Batistatou, A. & Greene, L.A. Induction of sulfated glycoprotein-2 (SGP-2) gene in PC12 cells by NGF and other treatments. Mol. Cell. Differen. 1:81-97, 1993.
- 15 86. Frohman, M.A., Dush, M.K. & Martin, G.R. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA 85:8998-9002, 1988.
- 20 87. Loh, E.Y., Elliott, J.F., Cwirla, S., Lanier, L.L. & Davis, M.M. Polymerase chain reaction with single-sided specificity: Analysis of T cell receptor δ chain. Science 243:217-220, 1989.
- 25 88. Ohara, O., Dorit, R.L. & Gilbert, W. One-sided polymerase chain reaction: the amplification of cDNA. Proc. Natl. Acad. Sci. USA 86:5673-5677, 1989.
- 30 89. Krieg, P. & Melton, D.A. Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNAs. Nucleic Acids Res. 12:7057-7070, 1984.
- 35 90. Tagawa, M., Sakamoto, T., Shigemoto, K., Matsubara, H., Tamura, Y., Ito, T., Nakamura, I., Okitsu, A., Imai, K. & Taniguchi, M. Expression of novel DNA-binding protein with zinc finger structure in various tumor cells. J. Biol. Chem.

265:20021-20026, 1990.

- 5 91. Goldstein, N.I., Nagle, R., Villar, H., Hersch, E. & Fisher, P.B. Isolation and characterization of a human monoclonal antibody which reacts with breast and colorectal carcinoma. Anticancer Res. 10:1491-1500, 1990.
- 10 92. Sambrook, J., Fritsch, E.F. & Maniatis, T. In: Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratories Press, Cold Spring Harbor, NY, 1989.
- 15 93. Su, Z.z., Grunberger, D. & Fisher, P.B. Suppression of adenovirus type 5 E1A-mediated transformation and expression of the transformed phenotype by caffeic acid phenyl ester (CAPE). Mol. Carcinogenesis 4:231-242, 1991.
- 20 94. Duigou, G.J., Su, Z.z., Babiss, L.E., Driscoll, B., Fung, Y-K.T. & Fisher, P.B. Analysis of viral and cellular gene expression during progression and suppression of the transformed phenotype in type 5 adenovirus-transformed rat embryo cells. Oncogene 25 6:1813-1824, 1991.
- 30 95. Su, Z.Z., Leon, J.A., Jiang, H., Austin, V.N., Zimmer, S.G. & Fisher, P.B. Wild-type adenovirus 5 transforming genes function as transdominant suppressors of oncogenesis in mutant adenovirus type 5 transformed rat embryo fibroblast cells. Cancer Res. 53:1929-1938, 1993.
- 35 96. Neckers, L., Whitesell, L., Rosolen, A. & Geselowitz, D.A. Antisense inhibition of oncogene

2174354

-204-

expression. Crit. Rev. Oncog. 3:175-231, 1992.

- 5 97. Grief, A.E. & Westphal, H. Antisense Myc sequences induce differentiation of F9 cells. Proc. Natl. Acad. Sci. USA 85:6806-6810, 1988.
- 10 98. Holt, J.T., Redner, R.L. & Nienhuis, A.W. An oligomer complementary to c-myc mRNA inhibits proliferation of HL-60 promyelocytic cells and induces differentiation. Mol. Cell. Biol. 8:963-973, 1988.
- 15 99. Prochownik, E.V., Kukowska, J. & Rogers, C. c-myc antisense transcripts accelerate differentiation and inhibit G1 progression in murine erythroleukemia cells. Mol. Cell. Biol. 8:3683-3695, 1988.
- 20 100. Yokoyama, K. & Imamoto, F. Transcriptional control of the endogenous MYC protooncogene by antisense RNA. Proc. Natl. Acad. Sci. USA 84:7363-7367, 1987.
- 25 101. Freytag, S.O. Enforced expression of c-myc oncogene inhibits cell differentiation by precluding entry into a distinct predifferentiation state in G0/G1. Mol. Cell. Biol. 8:1614-1624, 1988.
- 30 102. Toulme, J-J & Helene, C. Antimessenger oligodeoxyribonucleotides: an alternative to antisense RNA for artificial regulation of gene expression - a review. Gene 72:51-58, 1988.
- 35 103. Stein, C.A. & Cheng, Y-C. Antisense oligonucleotides as therapeutic agents - is the bullet really magical? Science 261:1004-1012, 1993.

21064354

104. Jaskulski, D., DeRiel, J.K., Mercer, E.M., Calabretta, B., & Baserga, R. Inhibition of cellular proliferation by antisense oligonucleotides to PCNA cyclin. *Science* 240:1544-1546, 1988.
- 5
105. Deiss, L.P. and Kimchi, A. A genetic tool used to identify thioredoxin as a mediator of a growth inhibitory signal. *Science* 252:117-120, 1991.
- 10
106. Izant, J.G. & Weintraub, H. Constitutive and conditional suppression of exogenous and endogeneous genes by anti-sense RNA. *Science* 229:345-352, 1985.
- 15
107. Vile, R.G. & Hart, I.R. In vitro and in vivo targeting of gene expression to melanoma cells. *Cancer Res.* 53:962-967, 1993.
- 20
108. Shen, R., Chen, Y., Huang, L., Vitale, E. & Solursh, M. Characterization of the human HOX-7 promoter and its regulation by retinoic acid. *Genes Develop*, in submission, 1993.
- 25
109. Shen, R., Goswami, S.K., Mascareno, E., Kumar, A. & Siddiqui, M.A.Q. Tissue-specific transcription of the cardiac myosin light-chain 2 gene is regulated by an upstream repressor element. *Mol. Cell. Biol.* 11:1676-1685, 1991.
- 30
110. Sanger, F., Nicklen, S. & Coulson, A.R. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463-5467, 1977.
- 35
111. McKnight, S.L. & Kingsbury, R. Transcriptional control signals of a eukaryotic protein-coding gene. *Science* 217:316-324, 1982.

WO 95/11986

PCT/US94/12160

-206-

112. Maniatis, T., Goodbourn, S. & Fischer, A.
Regulation of inducible and tissue-specific gene
expression. Science 236:1237-1244, 1987.
- 5 113. Ptashne, M. How eukaryotic transcriptional
activators work. Nature 335:683-689, 1988.
- 10 114. Su, Z.Z., Shen, R., O'Brian, C.A. & Fisher, P.B.
Induction of transformation progression in type 5
adenovirus-transformed rat embryo cells by a cloned
protein kinase C β_1 gene and reversal of progression
by 5-azacytidine. Oncogene 9: 1123-1132, 1994.
- 15 115. Dignam, J.D., Levbovitz, R.M. & Roeder, R.G.
Accurate transcription initiation by RNAPolymerase
II in a soluble extract from isolated mammalian
nuclei. Nuc. Acids Res. 11:1475-1489, 1982.

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Fourth Series of Experiments

mda-1: Novel gene which displays increased expression in IFN- β and IFN- β + MEZ treated H0-1 cells after 24 hours.
5 Decreased expression occurs in H0-1 cells treated with IFN- β + MEZ for 96 hours. (HJ 3-13).

TGGACTTGTGTTCTGACTAGAACTCAACATGTTACTAGGCACATGTGTCATGTCT
CAGGTCAGTGCTGTGACAGAATTGATACGAGAGAAATGTCGCTTATGCTATCACT
10 GATCTACACATGTCTGATAGATAGTCAGATACAGATGATGAGGAATCT (Seq. ID
No. 1)

mda-2:

15 GAATTCAGTGAACCTCTTTCTCATCTCTTTGTTTTGTGGCACTTCACAATGTAG
AGGAAAAAACCAAATGACCGCACTGTGATGTGAATGGCACCGAAGTCAGATGAGT
ATCCTGTAGGTCACCTGCAGCCTGGCTTGCCACTTGTCTTAACTCTGAATATTTT
ATTTCAAAGGTGCTAAAATCTGAAATCTGCTAGTGTGAACTTGCTCTACTCTCTG
AATGATTCAATCCTATTCATACTATCTTGTAGATATATCAACTAAAAAAA (Seq.
20 ID No. 2)

Properties of *mda-4*

25 This cDNA is novel (analysis of various gene data bases
indicates that the sequence of *mda-4* is 68.5% homologous
to the human interferon gamma induced protein).

Expression in H0-1 Human Melanoma Cells

30 Increased expression after 24 hour treatment of H0-1
cells with recombinant human fibroblast interferon (IFN- β)
(2,000 units/ml), mezerein (MEZ) (10 ng/ml) and to a
greater extent with the combination of IFN- β + MEZ (2,000
units/ml + 10 ng/ml).

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Analysis of terminally differentiated H0-1 cells, i.e., H0-1 cells treated with the combination of IFN- β + MEZ (2,000 units/ml + 10 ng/ml) for 96 hours, indicate continued increased expression in IFN- β + MEZ treated H0-1 cells.

Increased expression in H0-1 cells after 96 hour exposure to immune interferon (IFN- γ) (2,000 units/ml) and IFN- β + IFN- γ (1,000 units/ml + 1,000 units/ml) (note: this combination of agents results in a similar degree of growth suppression in H0-1 cells as does IFN- β + MEZ. However, growth suppression is reversible with the combination of interferons, whereas it is irreversible with the combination of IFN- β + MEZ).

mda-4 represents a novel IFN- γ -inducible gene which is also induced during terminal cell differentiation in H0-1 cells. Could prove useful as a gene marker for immune interferon response and as a gene marker for terminal differentiation in human melanoma cells.

Expression in Additional Human Melanoma Cells

Increased expression of *mda-4* results after a 24 hour treatment with IFN- β + MEZ in H0-1, L0-1, SH-1, WM278 and WM239 human melanoma cells. *Mda-4* is not expressed or inducible in the melanotic F0-1 human melanoma cell or in the C8161 human melanoma cells or C8161/6.3 cells (a C8161 human melanoma cell clone containing an inserted normal human chromosome 6: These cells are tumorigenic in nude mice, but unlike parental C8161 cells, they are non-metastatic).

Mda-4 displays increased expression in additional human melanoma cells besides the human melanoma cell from which it was cloned, i.e., H0-1 after 24 hour treatment with

IFN- β + MEZ.

5 Expression in Normal Cerebellum, a Central Nervous System
Tumor (Glioblastoma Multiforme) (GBM) and Normal Skin
Fibroblast Cell Lines

10 Mda-4 is not expressed de novo in normal cerebellum, GBM
or normal skin fibroblasts. However, it is inducible in
both normal cerebellum and GBM, but not in normal skin
fibroblasts, following a 24 hour treatment with IFN- β +
MEZ.

15 Mda-4 is susceptible to induction by IFN- β + MEZ in human
cerebellum and GBM cultures, but not in normal human skin
fibroblasts.

Expression in Colorectal Carcinoma (SW613), Endometrial
Adenocarcinoma (HTB113) and Prostate Carcinoma (LNCaP)

20 Mda-4 is not expressed de novo in various carcinoma cells
and it is not inducible in these cells following a 24
hour treatment with IFN- β + MEZ. Mda-4 is not expressed
in human carcinoma cells.

25 Effect of Various Treatment Protocols on Expression in
H0-1 Cells

30 A 24 hour treatment with IFN- β (2,000 units/ml), IFN- α
(2,000 units/ml), IFN- β + MEZ (2,000 units/ml + 10
ng/ml), IFN- α + MEZ (2,000 units/ml + 10 ng/ml), cis-
platinum (0.1 μ g/ml), gamma irradiation (treated with 3
gray and analyzed after 24 hours). In addition,
treatment with UV (10 joules/mm² and assayed 24 hours
later) results in increased expression in H0-1 cells.

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No or only a small change in expression is observed in H0-1 cells treated with MEZ (10 ng/ml; 24 hours or 96 hours), IFN- β (2,000 units/ml; 96 hours), phenyl butyrate (PB) (4 mM; treated for 24 hours, 4 days or 7 days),
5 mycophenolic acid (MPA) (3 μ M; 96 hours), trans retinoic acid (RA) (2.5 μ M; 24 hours), MPA + MEZ (3 μ M + 10 ng/ml; 96 hours), RA + MEZ (2.5 μ M; 96 hours), actinomycin D (5 μ g/ml for 2 hours, assayed after 24 hours), adriamycin (0.1 μ g/ml; 24 hours), vincristine (0.1 μ g/ml; 24 hours),
10 TNF- α (100 units/ml; 24 hours) or VP-16 (5 μ g/ml; 24 hours).

mda-4 is a novel gene which displays the following properties: 1) it is inducible in H0-1 cells during
15 terminal differentiation (treatment with IFN- β + MEZ for 96 hours) and following 96 hour treatment with recombinant gamma interferon (alone or in combination with IFN- β); 2) it is inducible by IFN- β + MEZ in a series of human melanomas in addition to H0-1, normal
20 cerebellum and GBM cells, but it is not expressed or inducible by IFN- β + MEZ in normal skin fibroblasts or three different types of carcinomas (colorectal, endometrial adenocarcinoma or prostate carcinoma); and 3) increased expression is induced in H0-1 cells treated
25 with specific DNA damaging agents (cis-platinum, gamma irradiation and UV irradiation).

This gene represents a cytokine-, DNA damage-, and chemotherapy- (cis-platinum) and terminal
30 differentiation-inducible gene possibly restricted to cells of neuroectodermal origin (melanoma and central nervous system). *mda-4* may prove useful: 1) as a marker for specific tissue lineage's (i.e., neuroectodermal) (diagnostic applications); 2) to monitor response to DNA
35 damage (induced by gamma irradiation and UV irradiation)

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and treatment with chemotherapeutic agents which work in a similar manner as cis-platinum (diagnostic applications); 3) to assay for types I (IFN- α and IFN- β) and type II (IFN- γ) interferon in biological fluids (diagnostic applications); and 4) to identify compounds which have the capacity to induce terminal differentiation in human melanoma cells (drug screening programs to identify new chemotherapeutic agents). Once full-length cDNAs are isolated, this gene (used in a sense orientation in an appropriate expression vector) may prove useful in inhibiting growth and inducing terminal differentiation in human melanomas and specific central nervous system tumors (GBM) (therapeutic applications). Antisense constructs of specific regions of this gene could also prove useful in preventing damage to normal tissue (e.g., bone marrow) treated with differentiation inducing and specific chemotherapeutic and DNA damaging agents (therapeutic applications).

20 mda-4

TTCTTCTTTGTAAAAGTTTTTAATACTGCTGAAAGATAAATTCATTCCAAAGA
GAATAATTATATAGCAAGATATTATCGGCACAGTGGTTTCTTAGAGGTAAATAGCG
CCTCACGTGTGTTAGATGCTGAATCTGACCAAA (SEQ ID No. 5)

25

Properties of mda-5

This cDNA is novel (it has sequence homology to a Homo sapiens putatively transcribed partial sequence; Accession number Z20545; from the UK-HGMP, MRC Human Genome Mapping Project Resource, Centre Clinical Research Centre, Watford Road, Harrow, Middlesex, HA1 England)

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Expression in H0-1 Human Melanoma Cells

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Increased expression after 24 hour treatment of H0-1 cells with recombinant human fibroblast interferon (IFN- β) (2,000 units/ml), and to a greater extent with IFN- β + MEZ (2,000 units/ml + 10 ng/ml) in combination.

5

Analysis of terminally differentiated H0-1 cells, i.e., H0-1 cells treated with the combination of IFN- β + MEZ (2,000 units/ml + 10 ng/ml) for 96 hours, indicate continued increased expression in IFN- β + MEZ treated H0-1 cells.

10

Enhanced expression of *mda-5* is also observed, albeit to a lesser extent, in H0-1 cells treated for 96 hours with immune interferon (IFN- γ) (2,000 units/ml) and in 96 hours IFN- β + IFN- γ (1,000 units/ml + 1,000 units/ml) treated H0-1 cells (greater increased than with IFN- γ alone) (note: this combination of agents results in a similar degree of growth suppression in H0-1 cells as does IFN- β + MEZ. However, growth suppression is reversible with this combination of interferons, whereas it is irreversible with the combination of IFN- β + MEZ).

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mda-5 represents a novel IFN- γ -inducible gene which also displays increased expression during terminal cell differentiation in H0-1 cells. This gene could prove useful as a marker for immune interferon response and as a marker for terminal differentiation in human melanoma cells.

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Expression in Additional Human Melanoma Cells

Increased expression of this gene occurs in H0-1, C8161, C8161/6.3 (a C8161 human melanoma cell clone containing an inserted normal human chromosome 6: These cells are tumorigenic in nude mice, but unlike parental C8161 cells

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they are non-metastatic), F0-1, L0-1, SH-1, WM278 and WM239 human melanoma cells treated with IFN- β + MEZ for 24 hours. This gene is constitutively expressed in immortalized human melanocytes FM5169 (transformed by SV40). Some upregulation is observed in FM5169 following IFN- β + MEZ treatment for 24 hours.

Expression of *mda-5* is increased by IFN- β + MEZ in 7 additional human melanoma cells besides the human melanoma cell it was cloned from i.e., H0-1. In addition, this gene is expressed in melanocytes and its expression is increased to a lesser degree than in most human melanomas following a 24 hour treatment with IFN- β + MEZ.

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Expression in Normal Cerebellum, a Central Nervous System Tumor (Glioblastoma Multiforme) (GBM) and Normal Skin Fibroblast Cell Lines

mda-5 is express de novo at low levels in normal cerebellum, but not in GBM or normal skin fibroblasts. However, expression is increased in normal cerebellum (>10-fold) and expression is induced in GBM (small induction) and normal skin fibroblasts (good induction) following a 24 hour treatment with IFN- β + MEZ.

This gene is susceptible to modulation by IFN- β + MEZ in human cerebellum, GBM and normal human skin fibroblasts. Differential de novo and inducible expression is seen in normal cerebellum cells versus GBM, with normal cerebellum displaying both higher de novo and inducible expression.

Expression in Colorectal Carcinoma (SW613), Endometrial Adenocarcinoma (HTB113) and Prostate Carcinoma (LNCaP)

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mda-5 is not expressed de novo in colorectal carcinoma (SW613) and endometrial adenocarcinoma (HTB113), whereas it is expressed at low levels in prostate carcinoma (LNCaP).

5

Following a 24 hour treatment with IFN- β + MEZ, mda-5 expression is induced at high levels in colorectal carcinoma (SW613) cells, but no expression is seen in endometrial adenocarcinoma (HTB113).

10

In the case of human prostate (LNCaP), IFN- β + MEZ treatment for 24 hours results in a 2- to 3-fold increase in mRNA expression.

15

This gene displays a differential pattern of both de novo and inducible expression in different human carcinomas.

Effect of Various Treatment Protocols on Expression in H0-1 Cells

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A 24 hour treatment with IFN- β (2,000 units/ml), IFN- α (2,000 units/ml), IFN- β + MEZ (2,000 units/ml + 10 ng/ml) and IFN- α + MEZ (2,000 units/ml + 10 ng/ml) results in increased expression in H0-1 cells.

25

mda-5 is induced after 96 hour treatment with IFN- γ and IFN- β + IFN- γ . Highest level of expression is observed in H0-1 cells treated with IFN- β + MEZ for 24 or 96 hours. Very low induction after 96 hour treatment with IFN- β (2,000 units/ml).

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No change in expression is observed in H0-1 cells treated with MEZ (10 ng/ml) (24 or 96 hours), MPA (3 μ M; 96 hours), RA (2.5 μ M; 96 hours), MPA + MEZ (3 μ M + 10 ng/ml; 96 hours), RA + MEZ (2.5 μ M + 10 ng/ml; 96 hours),

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phenyl butyrate (PB) (4 mM PB for 24 hours, 4 days or 7 days), cis-platinum (0.1 μ g/ml; 24 hours), gamma irradiation (treated with 3 gray and analyzed after 24 hours), UV (10 joules/mm², assayed 24 hours later),
5 actinomycin D (5 μ g/ml for 2 hours, assayed 24 hours later), adriamycin (0.1 μ g/ml; 24 hours), vincristine (0.1 μ g/ml; 24 hours), cis-platinum (0.1 μ g/ml; 24 hours), TNF- α (100 units/ml; 24 hours) or VP-16 (5 μ g/ml; 24 hours).

10

mda-5 is a novel gene which displays the following properties: 1) it is inducible during terminal differentiation (treatment with IFN- β + MEZ for 96 hours) and following treatment for 96 hours with recombinant
15 gamma interferon (alone or in combination with IFN- β); 2) treatment for 24 hours with IFN- β + MEZ results in increased expression in all human melanomas tested and in an SV40-immortalized human melanocyte; 3) it is highly inducible by IFN- β + MEZ within 24 hours in normal
20 cerebellum and normal skin fibroblast cells, but it is only weakly inducible in GBM; 4) it is differentially inducible in three different types of carcinomas (with induction greatest in colorectal, low induction in prostate carcinoma and no induction in endometrial
25 adenocarcinoma); and 5) increased expression is induced in H0-1 cells treated with both type I interferon (IFN- α and IFN- β) and type II interferon (IFN- γ) (IFN- β is more effective than IFN- α when used at an equivalent dose in enhancing expression of this gene).

30

This gene represents a cytokine- and terminal differentiation-inducible gene displaying increased expression in all melanomas, in select carcinomas, in normal skin fibroblasts and in both normal cerebellum and

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GBM. *mda-5* may be useful: 1) as a marker for specific tissue lineages and for distinguishing tumors of similar histotype (i.e., carcinomas) (diagnostic applications; 2) to monitor response to type I and II interferon treatment (diagnostic applications; and 3) to identify compounds which have the capacity to induce terminal differentiation in human melanoma cells (drug screening programs to identify new differentiation-inducing agents). Once full-length cDNAs are isolated, this gene (used in a sense orientation in an appropriate expression vector) may prove useful in inhibiting growth and inducing terminal differentiation in human melanomas and other classes of tumors (therapeutic applications).

15 *mda-5*

CTGCAAAGAAGTGTGCCGACTATAAATAAATGGTGAAATCATCTGCAAATGTGG
CCAGGCTTGGGGAACAATGATGGTGCACAAAGGCTTAGATTTGCCTTGTCTCAAA
ATAAGGAATTTTGTAGTGGTTTCAAAATATCACAAGAACGTACAAGTGGTAGATA
20 CTATCACATTCAGTACTATCAGAGTCG (SEQ ID No. 6)

ACAAACCAGTGATTCCCCTTCCTCAGATACTGGGACTAACAGCTTCACCTGGTGT
TGGAGGGGGCCACGAAGCAAGCCAAAGCTGAAGAACACATTTTAAACTATGTGCC
TATCTTGATGCATTTACTATTAAACTGTAAAGAAAACCTTGATCAACTGAAAA
25 ACCAAATACAGGAGCATGCAAGAAGTTTGCCATTGCAGATGCAACCAGAGAAGAT
CCATTAAAGAGAACTTCTAGAAATAATGACAAGGATTCAAACCTTATTGTCAAA
TGAGTCCAATGTCAGATTTTGGACTC (Seq. ID No. 7)

Properties of *mda-6*

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mda-6 is identical to WAF1, CIP1, SDI1 that encodes a Mr.21,000 protein (p21) that is an inhibitor of cyclin dependent kinases.

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Expression on H0-1 Human Melanoma Cells

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Increased expression after 24 hour treatment of H0-1 cells with recombinant human fibroblast interferon (IFN- β) (2,000 units/ml), MEZ (10 ng/ml) and to the greatest extent with IFN- β + MEZ (2,000 units/ml + 10 ng/ml) in combination.

Analysis of terminally differentiated H0-1 cells, i.e., H0-1 cells treated with the combination of IFN- β + MEZ (2,000 units/ml + 10 ng/ml) for 96 hours indicate continued increased expression in IFN- β + MEZ treated H0-1 cells; and (b) no significant or reduced alteration in expression after 96 hour treatment in H0-1 cells treated with IFN- β (2,000 units/ml), IFN- γ (2,000 units/ml), MEZ (10 ng/ml), mycophenolic acid (MPA) (3 μ M) (induces growth suppression increased melanin synthesis and morphology changes, but not terminal differentiation in H0-1 cells) trans retinoic acid (RA) (2.5 μ M) (increases melanin synthesis and tyrosinase activity, but does not alter growth, morphology or induce terminal differentiation in human melanoma cells), IFN- β + IFN- γ (1,000 units/ml + 1,000 units/ml) (growth suppressive without inducing markers of differentiation), MPA + MEZ (3 μ M + 10 ng/ml) (reversible growth suppression and induction of differentiation markers in H0-1 cells) and RA + MEZ (2.5 μ M + 10 ng/ml) (growth suppression and reversible induction of differentiation markers without inducing terminal cell differentiation).

mda-6 represents a novel gene which is enhanced in H0-1 cells by IFN- β + MEZ (which induces terminal differentiation) but not to the same extent by agents inducing growth suppression or various markers of differentiation. It could prove useful as a marker for the induction of terminal differentiation in human melanoma cells.

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Expression in Additional Human Melanoma Cells

Variable increases in expression of this gene occur in H0-1, C8161, C8161/6.3 (a C8161 human melanoma cell clone containing an inserted normal human chromosome 6: These cells are tumorigenic in nude mice, but unlike parental C8161 cells they are non-metastatic), F0-1, L0-1, SH-1, WM278 and WM239 human melanoma cells treated with IFN- β + MEZ for 24 hours. This gene is constitutively expressed in immortalized human melanocytes FM516-SV (transformed by SV40). Some upregulation is also observed in FM516-SV following IFN- β + MEZ treatment for 24 hours.

Expression of *mda-6* is increased by IFN- β + MEZ in 7 additional human melanoma cells besides the human melanoma cell it was cloned from, i.e., H0-1. In addition, this gene is expressed in melanocytes and its expression is increased following a 24 hour treatment with IFN- β + MEZ.

Expression in Normal Cerebellum, a Central Nervous System Tumor (Glioblastoma Multiforme) (GBM) and Normal Skin Fibroblast Cell Lines

mda-6 is expressed de novo at high levels in normal cerebellum and normal skin fibroblasts. *mda-6* is not expressed at significant levels in GBM cells. *mda-6* expression is increased in normal cerebellum (>10-fold) and expression is induced in GBM following a 24 hour treatment with IFN- β + MEZ. *mda-6* expression is not altered in normal human skin fibroblasts after a 24 hour treatment with IFN- β + MEZ.

This gene is susceptible to modulation by IFN- β + MEZ in

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human cerebellum and GBM. In contrast, this gene is expressed and no change in expression is seen following treatment in normal human skin fibroblasts. Differential de novo and inducible expression is also apparent in
5 normal cerebellum cells versus GBM, with normal cerebellum displaying higher de novo expression. This gene could be a component of growth control in central nervous system glial cells (including normal cerebellum cells), which is repressed in malignant GBM cells.

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Expression in Colorectal Carcinoma (SW613), Endometrial Adenocarcinoma (HTB112) and Prostate Carcinoma (LNCaP)

mda-6 is expressed at high levels de novo in colorectal
15 carcinoma (SW613) and prostate carcinoma (LNCaP). *mda-6* de novo expression in endometrial adenocarcinoma (HTB113) is low. Treatment for 24 hours with IFN- β + MEZ does not significantly alter *mda-6* expression in colorectal carcinoma (SW613) or prostate carcinoma (LNCaP).
20 Treatment for 24 hours with IFN- β + MEZ induces *mda-6* expression in endometrial adenocarcinoma (HTB113) to a similar level as in colorectal and prostate carcinomas.

mda-6 displays a differential pattern of both de novo and
25 inducible expression in different human carcinomas. De novo expression is low in endometrial adenocarcinoma and high in colorectal and prostate carcinoma. Inducible expression following treatment with IFN- β + MEZ is only observed in endometrial adenocarcinoma cells.

30

Effect of Various Treatment Protocols on Expression in H0-1 Cells

Treatment with IFN- β (2,000 units/ml; 24 hours), MEZ (10
35 ng/ml; 24 hours), IFN- β + MEZ (2,000 units/ml + 10 ng/ml;

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24 hours), actinomycin D (5 μ g/ml; 2 hour treatment followed by 24 hour growth), adriamycin (0.1 μ g/ml; 24 hour) and VP-16 (5 μ g/ml; 24 hour) results in increased expression in H0-1 cells. Highest level of induction observed in H0-1 cells treated with IFN- β + MEZ for 24 hours or 96 hours; and actinomycin D, adriamycin and VP-16 treated for 24 hours.

Decreased expression of *mda-6* is observed in H0-1 cells treated with phenyl butyrate (PB) (4 mM) (24 hours, 4 or 7 days), cis-platinum (0.1 μ g/ml; 24 hours), UV (10 joules/mm²; 2 hours after treatment), gamma irradiation (3 gray; assayed after 24 hours), IFN- α (2,000 units/ml; 24 hours) and TNF- α (100 units/ml; 24 hours).

No change in *mda-6* expression observed in H0-1 cells treated with UV (10 joules/mm²) and assayed after 14 or 24 hours and in H0-1 cells treated for 24 hours with IFN- α + MEZ (2,000 units/ml + 10 ng/ml), or vincristine (0.1 μ g/ml; 24 hours).

mda-6 is a novel gene which displays the following properties: 1) its expression is increased during terminal differentiation (treatment with IFN- β + MEZ for 96 hours); 2) treatment for 24 hours with IFN- β + MEZ results in variable increases in its expression in all human melanomas tested and in an SV40-immortalized human melanocyte; 3) it is expressed in early stage melanomas (radial and early vertical growth phase melanomas), but not or at reduced levels in more advanced melanomas (metastatic melanomas); 4) it is expressed de novo and highly inducible by IFN- β + MEZ within 24 hours in normal cerebellum; 5) it is not expressed de novo in GBM and only marginally induced in GBM after 24 hour treatment

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with IFN- β + MEZ; 6) high levels of de novo expression are seen in normal skin fibroblasts, colorectal carcinoma (SW613) and prostate carcinoma (LNCaP), but IFN- β + MEZ treatment does not significantly alter expression; 7) endometrial adenocarcinoma (HTB113) cells display low levels of expression of this gene, whereas IFN- β + MEZ treatment for 24 hours results in high levels of expression; 8) expression is increased in H0-1 cells treated with actinomycin D, adriamycin and VP-16; 9) expression is reduced in H0-1 cells treated with phenyl butyrate, gamma irradiation, cis-platinum and TNF- α ; and 10) expression is highest in normal melanocytes and a dysplastic nevus and reduced in radial growth phase (RGP) and vertical growth phase (VGP) primary melanomas and lowest in metastatic melanoma; 11) expression is reduced as a function of tumorigenic progression in Matrigel-progressed RGP and early VGP primary melanoma: 12) expression is low in tumorigenic and metastatic C8161 human melanoma cells and increased in three independent C8161 clones containing an inserted normal chromosome 6 that are tumorigenic but not metastatic: 13) an immediate early response gene i.e., induced in the presence of cycloheximide, in human promyelocytic leukemia. HL-60 cells induced to differentiate into monocytes and macrophages (treatment with 12-O-tetradecanoyl-phorbol-13-acetate (TPA) or vitamin D3) or granulocytes (treatment with all-trans retinoic acid (RA) or dimethyl sulfoxide (DMSO)); 14) induced as a function of growth arrest and differentiation in human neuroblastoma cells by treatment with the combination of phenylacetate and RA; and 15) induced during the induction of differentiation and growth arrest in human histiocytic lymphoma, U-937, cells by treatment with TPA.

mda-6 represents a terminal differentiation-regulated

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gene displaying increased expression in all melanomas tested, in specific carcinomas, in normal cerebellum cells and in GBM cells treated with IFN- β + MEZ. *mda-6* is also induced during the induction of monocyte/macrophage and granulocyte differentiation in human promyelocytic leukemia (HL-60) cells; differentiation in human neuroblastoma cells; and differentiation in histiocytic lymphoma (U-937) cells. This gene also displays increased expression in cells treated with specific chemotherapeutic agents, including adriamycin and VP-16. In contrast, expression of *mda-6* is decreased following treatment with gamma irradiation, the demethylating anticancer agent phenyl butyrate, the cytokine TNF- α and the chemotherapeutic agent cis-platinum. *mda-6* may be useful: 1) as a marker for specific tissue lineages and for distinguishing tumors of similar histotype (i.e., carcinomas, astrocytomas) (diagnostic applications); 2) to monitor response to topoisomerase inhibitors such as VP-16 and specific chemotherapeutic agents which function in a similar manner as adriamycin and cis-platinum (drug screening programs to identify new chemotherapeutic agents); 3) to identify compounds which have the capacity to induce terminal differentiation in human melanoma cells myeloid leukemic cells, histiocytic lymphoma and neuroblastoma (drug screening programs to identify new differentiation-inducing and chemotherapeutic agents); and 4) to monitor states of tumor progression, i.e., only expressed or expressed at higher levels in less aggressive and early stage cancers. Once full-length cDNAs are isolated, this gene (used in a sense orientation in an appropriate expression vector) may also prove useful in inhibiting growth and inducing terminal differentiation in human melanomas and other classes of tumors (therapeutic applications). Overexpression of this gene in specific

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cell types (such as bone marrow cells) may also result in a decreased sensitivity of these cells to various DNA damaging agents and chemotherapeutic agents (therapeutic applications). This could prove useful in protecting bone marrow cells from damage induced by radiation and chemotherapy (therapeutic applications). Similarly, use of antisense constructs may also result in a decreased sensitivity to growth suppression in normal cells induced by specific classes of DNA damaging and therapeutic agents (therapeutic applications). This gene may also prove useful in the classification of more advanced astrocytomas (such as GBM) from less advanced earlier stages of astrocytomas (diagnostic applications). This gene may also prove useful in distinguishing between early stage (early radial growth phase, early vertical growth phase) melanoma and late stage (late vertical growth phase, metastatic) melanoma (diagnostic applications).

20 mda-6

ATGCCACGTGGGCTCATATGGGGCTGGGAGTAGTTGTCTTTCCTGGCACTAACGT
TGAGCCCCTGGAGGCACTGAAGTGCTTAGTGTACTTGGAGTATTGGGGTCTGACC
CAAACACCTTCCAGCTCCTGTAACATACTGGCCTGGACTGTTTTCTCTCGCGCCT
25 CCCCATGTGCTCCTGGTTCCCGTTTCCTCCACCTAGACTGTAAACCTCTCGCA
(SEQ ID No. 8)

CCTGCAGTCCTGGAAGCGCGAGGGCCTCAAACGCGCTCTACATCTTCTGCCTTAG
TCTCAGTTTGCGTGTCTTAATTATTATTTGTGTTTTTAATTAAACACCTCCTCAT
30 GTACATAACCCTGGCCGCCCCCTGCCCCCAGCCTCTCGGATTAGAATTATTTAAA
CAAAAACCTAGGCGGTTGAATGAGAGGTTCTATGAGTACTGGGCATTTTTATTTT
ATGAAATACTATTTAAAGCCTCCTCATCCCATGTTCTCCTTTTCCTCTCTCCCGG
AGTT (Seq. ID No. 9)

35 Properties of mda-7

mda-7 is a novel cDNA (it has no sequence homology with previously reported genes in the various DNA data bases).

Expression in H0-1 Human Melanoma Cells

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Increased expression of *mda-7* after 24 hour treatment of H0-1 cells with recombinant human fibroblast interferon (IFN- β) (2000 units/ml), MEZ (10 ng/ml) and to the greatest extent with IFN- β + MEZ (2000 units/ml + 10 ng/ml).

10

Increased expression of *mda-7* is observed in H0-1 cells treated for 96 hours with IFN- β (2000 units/ml), MEZ (10 ng/ml), MPA (3 μ M), IFN- β + IFN- γ (1000 units/ml + 1000 units/ml), IFN- β + MEZ (2000 units/ml + 10 ng/ml), MPA + MEZ (3 μ M + 10 ng/ml) and RA + MEZ (2.5 μ M + 10 ng/ml). Maximum induction is observed with IFN- β + MEZ followed by MPA + MEZ and IFN- β + IFN- γ .

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The relative level of *mda-7* induction correlates with the degree of growth suppression observed H0-1 cells treated with the various growth and differentiation modulating agents. The greatest increase in expression is observed in cells induced to irreversibly lose proliferative capacity and become terminally differentiated by treatment with IFN- β + MEZ.

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Expression in Additional Human Melanoma Cells

Increased expression of *mda-7* occurs in H0-1, C8161, C8161/6.3 (a C8161 human melanoma cell clone containing an inserted normal human chromosome 6: These cells are tumorigenic in nude mice, but unlike parental C8161 cells they are non-metastatic), F0-1, L0-1, SH-1, WM278 and WM239 human melanoma cells treated with IFN- β + MEZ for

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24 hours. This gene is constitutively expressed in immortalized human melanocytes FM5169 (transformed by SV40). However, no increase in expression is observed in FM5169 following IFN- β + MEZ treatment for 24 hours.

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mda-7 is either variably expressed or variably induced in all human melanoma cells treated with IFN- β + MEZ. In contrast, although this gene is expressed in melanocytes, no change in expression is observed following a 24 hour treatment with IFN- β + MEZ.

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Expression in Normal Cerebellum, a Central Nervous System Tumor (Glioblastoma Multiforme) (GBM) and Normal Skin Fibroblast Cell Lines

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mda-7 is not expressed de novo in normal cerebellum, GBM or normal skin fibroblasts.

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Expression of *mda-7* is induced in normal cerebellum, GBM and normal skin fibroblasts following a 24 hour treatment with IFN- β + MEZ.

25

mda-7 is not expressed de novo but is susceptible to induction by IFN- β + MEZ in human cerebellum, GBM and normal human skin fibroblasts.

Expression in Colorectal Carcinoma (SW613), Endometrial Adenocarcinoma (HTB113) and Prostate Carcinoma (LNCaP)

30

mda-7 is not expressed de novo in colorectal carcinoma (SW613), endometrial adenocarcinoma (HTB113) or prostate carcinoma (LNCaP).

35

mda-7 is not induced in colorectal carcinoma (SW613), endometrial adenocarcinoma (HTB113) or prostate carcinoma

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(LNCaP) cells following a 24 hour treatment with IFN- β + MEZ.

5 This gene is neither expressed de novo or inducible by IFN- β + MEZ in human carcinomas.

Effect of Various Treatment Protocols on Expression in H0-1 Cells

10 Treatment with IFN- β (2000 units/ml; 24 hours), MEZ (10 ng/ml; 24 hours), IFN- β + MEZ (2000 units/ml + 10 ng/ml; 24 hours and 96 hours), IFN- α + MEZ (2000 units/ml + 10 ng/ml; 24 hours), adriamycin (0.1 μ g/ml; 24 hours), vincristine (0.1 μ g/ml; 24 hours), and UV (10 joules/mm²
15 and assayed 24 hours later) results in increased *mda-7* expression in H0-1 cells. *mda-7* is also induced after 96 hour treatment with MPA (3 μ M), IFN- β + IFN- γ (1000 units/ml + 1000 units/ml), MPA + MEZ (3 μ M + 10 ng/ml) and RA + MEZ (2.5 μ M + 10 ng/ml). Highest level of
20 expression observed in H0-1 cells treated with IFN- β + MEZ for 24 or 96 hours.

No induction in *mda-7* expression is observed in H0-1 cells treated with IFN- α (2000 units/ml; 24 hours), IFN- γ
25 (2000 unit/ml; 96 hours), phenyl butyrate (4 mM PB for 24 hours, 4 days or 7 days), cis-platinum (0.1 μ g/ml; 24 hours), gamma irradiation (treated with 3 gray and analyzed after 24 hours), actinomycin D (5 μ g/ml for 2 hours, assayed 24 hours later), TNF- α (100 units/ml; 24
30 hours) or VP-16 (5 μ g/ml; 24 hours).

mda-7 is a growth and differentiation and senescence-regulated novel gene which displays the following properties: 1) it is inducible during terminal

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differentiation (treatment with IFN- β + MEZ for 96 hours) and following treatment for 96 hours with many growth modulating and differentiation inducing agents; 2) treatment for 24 hours with IFN- β + MEZ results in increased expression in all human melanomas tested, but not in an SV40-immortalized human melanocyte; 3) it is not expressed de novo but it is highly inducible by IFN- β + MEZ within 24 hours in normal cerebellum, GBM and normal skin fibroblast cells; 4) it is not expressed or inducible in colorectal, endometrial or prostate carcinomas; 5) increased expression is induced in H0-1 cells treated with adriamycin, vincristine and UV irradiation; and 6) it is not expressed in growing human neuroblastoma cells but it is inducible following growth suppression and the induction of terminal differentiation; 7) it is not expressed in human promyelocytic leukemia (HL-60 and human histiocytic lymphoma (U-937) cells but it is induced following the induction of growth arrest and terminal differentiation; and 8) it is not expressed in actively growing human cells but it is induced during cellular senescence.

mda-7 is a novel growth- and terminal differentiation-regulatable gene displaying increased expression in all melanomas (but not in melanocytes), and in normal skin fibroblasts and in both normal cerebellum and GBM cells treated with IFN- β + MEZ. In contrast, *mda-7* is not expressed or induced in a series of carcinomas. *mda-7* may be useful: 1) as a marker for specific tissue lineage's (i.e., melanomas from keratinocytes) (diagnostic applications); 2) in distinguishing fibroblasts (inducible with IFN- β + MEZ) from carcinomas (non-inducible with IFN- β + MEZ) (diagnostic applications); 3) for the identification of agents capable of inducing growth suppression and various

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components of the differentiation process (including terminal differentiation) in human melanomas (drug screening programs to identify new differentiation-inducing and chemotherapeutic agents); and 4) distinguishing melanocytes, and perhaps nevi, from early and late stage melanoma cells (diagnostic applications). Once full-length cDNAs are isolated, this gene (used in a sense orientation in an appropriate expression vector) may also prove useful in inhibiting growth and inducing terminal differentiation in human melanomas (therapeutic applications).

mda-7

CAGAATATTGTGCCCCATGCTTCTTTACCCCTCACAATCCTTGCCACAGTGTGGG
CAGTGGATGGGTGCTTAGTAAGTACTTAATAAACTGTGGTGCTTTTTTTGGCCTG
TCTTTGGATTGTTAAAAAACAGAGAGGGATGCTTGGATGTAAACTGAACTTCAGA
GCATGAAATCACACTGTCTCTGATATCT (SEQ ID No. 10)

Properties of mda-8

mda-8 is a novel cDNA (it has no sequence homology with previously reported genes in the various DNA data bases)

Expression in H0-1 Human Melanoma Cells

Increased expression of *mda-8* results in H0-1 cells after 24 hour treatment with the combination of IFN- β + MEZ (2000 units/ml + 10 ng/ml).

30

Analysis of terminally differentiated H0-1 cells, i.e., H0-1 cells treated with the combination of IFN- β + MEZ (2000 units/ml + 10 ng/ml) for 96 hours indicate continued increased expression of *mda-8*.

35

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Treatment of H0-1 cells for 96 hours with immune interferon (IFN- γ) (2000 units/ml) or IFN- β + IFN- γ (1000 units/ml + 1000 units/ml) results in enhanced *mda-8* expression. The level of increased *mda-8* expression at 96 hour is similar in IFN- γ , IFN- β + IFN- γ and IFN- β + MEZ treated H0-1 cells. (Note: The combination of IFN- β + IFN- γ results in a similar degree of growth suppression at 96 hour in H0-1 cells as does IFN- β + MEZ. However, growth expression is reversible with the combination of interferons, whereas it is irreversible with the combination of IFN- β + MEZ).

mda-8 is a novel IFN- γ -inducible gene which also displays increased expression during terminal cell differentiation in H0-1 human melanoma cells. *mda-8* could prove useful as a marker for immune interferon response and a marker for terminal differentiation in human melanoma cells.

Expression in Additional Human Melanoma Cells

20

Increased expression of *mda-8* occurs in H0-1, C8161 and WM278 human melanoma cells treated for 24 hours with IFN- β + MEZ (2000 units/ml + 10 ng/ml).

No change in expression of *mda-8* is seen in additional human melanomas treated for 24 hours with IFN- β + MEZ, including C8161/6.3 (a C8161 human melanoma cell clone containing an inserted normal human chromosome 6: These cells are tumorigenic in nude mice, but unlike parental C8161 cells they are non-metastatic), F0-1, L0-1, SH-1, and WM239.

Expression of this gene is increased by IFN- β + MEZ in specific human melanoma cells.

35

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Expression in Normal Cerebellum, a Central Nervous System Tumor (Glioblastoma Multiforme) (GBM) and Normal Skin Fibroblast Cell Lines

5 *mda-8* is expressed de novo in normal cerebellum, but not in GBM.

mda-8 is expressed de novo in normal skin fibroblasts.

10 Growth for 24 hours in IFN- β + MEZ (2000 units/ml + 10 ng/ml) results in marginal changes in *mda-8* expression in normal cerebellum and normal skin fibroblasts.

15 Expression of *mda-8* is induced at high levels in GBM cells following a 24 hour exposure to IFN- β + MEZ.

20 This gene is expressed de novo in both normal cerebellum and normal skin fibroblasts, but not in GBM. This gene is induced by IFN- β + MEZ in human GBM, but expression is not altered in normal cerebellum cells and normal skin fibroblasts.

Expression in Colorectal Carcinoma (SW613), Endometrial Adenocarcinoma (HTB113) and Prostate Carcinoma (LNCaP)

25

mda-8 is expressed de novo in colorectal carcinoma (SW613), endometrial adenocarcinoma (HTB113) and prostate carcinoma (LNCaP).

30 Following a 24 hour treatment with IFN- β + MEZ, expression of *mda-8* is unaffected in colorectal carcinoma (SW613), endometrial adenocarcinoma (HTB113) and prostate carcinoma (LNCaP) cells.

35 *mda-8* is expressed de novo in the three types of

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carcinomas. *mda-8* gene expression is not altered in the three carcinomas after treatment for 24 hours with IFN- β + MEZ.

5 Effect of Various Treatment Protocols on Expression in
 H0-1 Cells

Increased expression of *mda-8* results after treatment with IFN- β (2000 units/ml; 24 hours), actinomycin D (5
10 μ g/ml for 2 hours, assayed 24 hours later), adriamycin (0.1 μ g/ml; 24 hours), cis-platinum (0.1 μ g/ml; 24 hours) and UV (10 joules/mm², assayed 2, 14 and 24 hours later).

A 96 hour treatment with IFN- γ (2000 units/ml), IFN- β +
15 MEZ (2000 units/ml) and IFN- β + IFN- γ (1000 units + 1000 units) results in increased *mda-8* expression.

No change in expression of *mda-8* is observed in H0-1 cells treated with MEZ (10 ng/ml; 24 or 96 hours), IFN- β
20 (2000 units/ml; 24 or 96 hours), MPA (3 μ M; 96 hours), RA (2.5 μ M; 96 hours), MPA + MEZ (3 μ M + 10 ng/ml; 96 hours), RA + MEZ (2.5 μ M + 10 ng/ml), phenyl butyrate (4 mM PB for 24 hours, 4 days or 7 days), gamma irradiation (treated with 3 gray and analyzed after 24 hours),
25 vincristine (0.1 μ g/ml; 24 hours), TNF- α (100 units/ml; 24 hours), VP-16 (5 μ g/ml; 24 hours), IFN- α (2000 units/ml) or IFN- α + MEZ (2000 units/ml + 10 ng/ml).

mda-8 is a novel gene which displays the following
30 properties: 1) it is inducible during terminal differentiation (treatment with IFN- β + MEZ for 96 hours) and following treatment for 96 hours with recombinant gamma interferon (alone or in combination with IFN- β); 2) treatment for 24 hours with IFN- β + MEZ results in

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increased expression in only select human melanomas; 3) it is expressed de novo in normal cerebellum, normal skin fibroblasts, colorectal carcinoma (SW613), endometrial adenocarcinoma (HTB113) and prostate carcinoma (LNCaP), but not in GBM; 4) treatment with IFN- β + MEZ for 24 hours results in no change in expression in normal cerebellum, normal skin fibroblasts, colorectal carcinoma (SW613), endometrial adenocarcinoma (HTB113) and prostate carcinoma (LNCaP); 5) treatment for 24 hours with IFN- β + MEZ induces expression in GBM cells; and 6) increased expression is induced in H0-1 cells treated with actinomycin D, adriamycin, cis-platinum and UV irradiation.

mda-8 is a cytokine- and terminal differentiation-responsive gene displaying increased expression in specific human melanomas and GBM cells treated with IFN- β + MEZ (also inducible in H0-1 after 96 hour treatment with IFN- γ and IFN- γ + IFN- β). Enhanced expression is also apparent in H0-1 human melanoma cells treated with the transcription inhibitor actinomycin D, the chemotherapeutic agents adriamycin and cis-platinum and UV irradiation. *mda-8* may be useful: 1) as a marker for distinguishing between normal glial cells and malignant astrocytomas (such as GBM) (diagnostic applications); 2) to monitor response to type II interferon treatment (diagnostic applications); and 3) to identify compounds which have the capacity to induce terminal differentiation, induce similar cytotoxic effects as adriamycin, cis-platinum and UV irradiation (drug screening programs to identify new differentiation-inducing and chemotherapeutic agents). Once full-length cDNAs are isolated, this gene (used in a sense orientation in an appropriate expression vector) may prove useful in inhibiting growth and inducing terminal

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differentiation in specific human melanomas and glioblastoma multiforme tumors (therapeutic applications).

5 mda-8

TTAAAGTTTGCCCTTGTGCTAAAGTGCCAGTGTATGTATGTTATACTTGATTTGG
TTGTAAACTATATTTCAAAGTAAACCCTAGTGTAATAAGTTTTATAACTAAAAAG
GTTTAAGCTGCTAAAACTATTTTTTAAGAGATGTGAAATCGAGTATGGGACTATCT
10 TTTTTCCTCCTCTAAA (SEQ ID No. 11)

Properties of mda-9

mda-9 is a novel cDNA (it displays sequence homology to
15 human transforming growth factor- β (TGF- β) mRNA, 55.1%
homology in 138 bp; GB-Pr:Humtgfb).

Expression in H0-1 Human Melanoma Cells

20 Increased expression of mda-9 occurs after 24 hour
treatment of H0-1 cells with the combination of IFN- β +
MEZ (2000 units/ml + 10 ng/ml).

Increased expression of mda-9 persists in terminally
25 differentiated H0-1 cells, i.e., H0-1 cells treated with
the combination of IFN- β + MEZ (2000 units/ml + 10 ng/ml)
for 96 hours.

mda-9 is a novel gene (with homology to TGF- β) which
30 displays increased expression in terminally
differentiated H0-1 human melanoma cells.

Expression in Additional Human Melanoma Cells

35 Variable increases in expression of mda-9 occurs in H0-1

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and C8161 human melanoma cells treated for 24 hours with IFN- β + MEZ (2000 units/ml + 10 ng/ml).

5 The level of expression of *mda-9* decreases in SH-1 cells treated for 24 hours with IFN- β + MEZ (2000 units/ml + 10 μ g/ml).

10 No change in *mda-9* expression results in FO-1, LO-1 or C8161/6.3 cells (a C8161 human melanoma cell clone containing an inserted normal human chromosome 6: These cells are tumorigenic in nude mice, but unlike parental C8161 cells they are non-metastatic).

15 Expression of *mda-9* is increased by IFN- β + MEZ in specific human melanoma cells. The lack of enhanced expression in C8161/6.3 cells treated with IFN- β + MEZ, whereas parental C8161 cells do show an increase, suggests that modulation of this gene may correlate with more advanced stages of melanoma development (i.e.,
20 melanoma cells with metastatic potential).

Effect of Various Treatment Protocols on Expression in H0-1 Cells

25 Increased expression of *mda-9* is observed in H0-1 cells treated with IFN- β (2000 units/ml; 24 hours), MEZ (10 ng/ml; 24 hours), IFN- β + MEZ (2000 units/ml + 10 ng/ml; 24 and 96 hours), phenyl butyrate (4 mM PB for 24 hours, 4 days or 7 days), gamma irradiation (treated with 3 gray
30 and analyzed after 24 hours), TNF- α (100 units/ml; 24 hours), IFN- α (2000 units/ml), IFN- α + MEZ (200 units/ml + 10 ng/ml), VP-16 (5 μ g/ml; 24 hours) or UV (10 joules/mm², assayed 2 or 14 hours later).

35

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No change in *mda-9* expression is observed in H0-1 cells treated with actinomycin D (5 μ g/ml for 2 hours, assayed 24 hours later), UV (10 joules/mm², assayed 24 hours later), cis-platinum (0.1 μ g/ml; 24 hours), vincristine (0.1 μ g/ml; 24 hours), IFN- β (2000 units/ml; 96 hours), IFN- γ (2000 units/ml; 96 hours), MEZ (10 ng/ml; 96 hours), MPA (3 μ M; 96 hours), RA (2.5 μ M; 96 hours), IFN- β + IFN- γ (1000 units/ml + 1000 units/ml; 96 hours), MPA + MEZ (3 μ M + 10 ng/ml; 96 hours) or RA + MEZ (2.5 μ M + 10 ng/ml).

mda-9 is a novel gene with sequence homology to TGF- β which displays the following properties: 1) it is inducible during terminal differentiation (treatment with IFN- β + MEZ for 96 hours) in H0-1 human melanoma cells; 2) treatment for 24 hours with IFN- β + MEZ results in increased expression in several human melanomas; 3) treatment for 24 hours with IFN- β + MEZ results in increased expression in the tumorigenic and metastatic human melanoma C8161, but not in C8161/6.3 which is tumorigenic but not metastatic; and 4) increased expression is induced in H0-1 cells treated with a number of agents including phenyl butyrate, gamma irradiation, TNF- α , UV irradiation (after 2 and 14 hours, but not after 24 hours), IFN- α and IFN- α + MEZ.

mda-9 is a terminal differentiation-responsive gene displaying increased expression in several human melanomas treated with IFN- β + MEZ. Enhanced expression is induced by IFN- β + MEZ in the tumorigenic and metastatic human melanoma cell C8161, but not its reverted derivative C8161/6.3 (which retains tumorigenicity, but has lost metastatic potential). Increased expression is also apparent in H0-1 human

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melanoma cells treated with the demethylating anticancer agent phenyl butyrate, the cytokine $\text{TNF-}\alpha$, gamma irradiation and UV irradiation.

5 *mda-9* may be useful: 1) as a marker for distinguishing between early stage and more progressed human melanoma (diagnostic applications); and 2) to identify compounds which have the capacity to induce terminal differentiation and to induce specific patterns of DNA
10 damage as induced by UV irradiation and gamma irradiation (drug screening programs to identify new differentiation-inducing and chemotherapeutic agents). Once full-length cDNAs are isolated, this gene (used in a sense orientation in an appropriate expression vector) may also
15 prove useful in inhibiting growth and inducing terminal differentiation in specific human melanomas (therapeutic applications). When used in an antisense orientation, expression of this gene might allow normal cells (such as bone marrow cells) to be engineered to be resistant to
20 cytotoxicity induced by specific chemotherapeutic agents and gamma irradiation (therapeutic applications).

mda-9

25 AAAACTTTCAAGAGATTTACTGACTTTCCTAGAATAGTTTCTCTACTGGAAACCT
GATGCTTTTATAAGCCATTGTGATTAGGATGACTGTTACAGGCTTAGCTTTGTGT
GAAAACCAGTCACCTTTCTCCTAGGTAATGAGTAGTGCTGTTTCATATTACTTTAG
TTCTATAGCATACTCGATCTTTAACATGCTATCATAGTACATTAGATGATG (SEQ
ID No. 12)

30

Additional *mda* Genes Isolated Using Subtraction
Hybridization from H0-1 Human Melanoma Cells Treated with
IFN- β + MEZ

35 *mda-1*: Novel gene which displays increased expression in

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IFN- β and IFN- β + MEZ treated H0-1 cells after 24 hours (HP 2-36). (Jiang and Fisher, Molecular and Cellular Differentiation, 1 (3), 285-299, 1993).

5 mda-2: Novel gene which displays increased expression in IFN- β and IFN- β + MEZ treated H0-1 cells after 24 hours (HP-3-31). (Jiang and Fisher, Molecular and Cellular Differentiation, 1 (3), 285-299, 1993).

10 mda-3: Increased expression in MEZ and IFN- β + MEZ treated H0-1 cells after 24 hours (HP 2-4). (Identical to Human GOS 19-1 mRNA, cytokine (Gb-Pr:Hummipla), human TPA-inducible mRNA, pLD78 (GB-Pr:Humpld78). (Jiang and Fisher, Molecular and Cellular Differentiation, 1 (3), 15 285-299, 1993).

mda-11: Novel gene which displays increased expression in IFN- β + MEZ treated H0-1 cells after 24 hours. (HJ 2-78). (87.2% identity to the rat ribosomal protein 20 IF116).

CGCACGTCACCCACCTTCCGGCGGCCGAAGACACTGCGACTCCGGAGACAGCCCA
AATATCCTCGGAAGAGCGCTCCCAGGAGAAACAAGCTTGACCACTATGCTATCAT
CAAGTTTCCGCTGACCACTGAGTCTGCCATGAAGAAGATAGAAGACAACAACACA
25 CTTGTGTTCATTGTGGATGTTAAAGCCAACAAGCACCAGATTAACAGCTGTGAGA
GCTGTATGACATTGATGTGCAGTACACCTGATCGTCT (Seq. ID No. 13)

mda-12: Gene which displays increased expression in IFN- β + MEZ treated H0-1 cells after 24 hours. (HP 3-8).
30 (Identical to Human GOS19-3 mRNA (Gb-Humcpgcus2), LD78A (Gb-Pr:Humld78a).

mda-13: Gene which displays increased expression in IFN- β and IFN- β + MEZ treated H0-1 cells after 24 hours. (HP
35 S-7). (Identical to interferon stimulated gene -56

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(ISG56), an IFN- β inducible gene).

5 *mda-14*: Gene which displays increased expression in IFN- β + MEZ treated H0-1 cells after 24 hours. (HP 2-59 and HP 3-114, same gene isolated independently two times). (Identical to interleukin-8 (IL-8) (Gb-Un:M28130), human mRNA for MDNCF (monocyte derived neutrophil chemotactic factor) (Gb-Pr:Nummdncf).

10 TAAAAAATTCATTCTCTGTGGTATCCAAGAATCAGTGAAGATGCCAGTGAAACT
TCAAGCAAATCTACTTCAACACTTCATGTATTGTGTGGGTCTGTTGTAGGGTTGC
CAGATGCAATACAAGATTCCTGGTTAAATTTGAATTTTCAGTAAACAATGAATAGT
TTTTCATTGTACATGAAATATCAGAACATACTTATATGTAAGTATATTATTGATG
ACAAACACAATATTTAATATA (Seq. ID No. 14)

15

mda-15: Gene which displays increased expression in IFN- β + MEZ treated H0-1 cells after 24 hours (HP 2-64). (Identical to vimentin, intermediate filament protein (Gb-Pr:Humviment).

20

mda-16: Gene which displays increased expression in IFN- β + MEZ treated H0-1 cells after 24 hours. (HP 2-18). (Identical to human apoferritin H gene (Gb-Pr:Humferg2).

25

mda-17: Gene which displays increased expression in IFN- β + MEZ treated H0-1 cells after 24 hours. (HP 2-40). (Identical to IFP-53 (Gb Pr:Humifp), IFN-inducible gamma 2 protein (Gb-Huminfig).

30

GGGGGTGAAACTTTCCAGTTTACTGAACTCCAGACCATGCATGTAGTCCACTCCA
GAAATCATGCTCGCTTCCTTGGCACACAGTGTTCCTGCCAAATGACCCTAGAC
CCTCTGTCCTGCAGAGTCAGGGTGGCTTTTACCCTGACTGTGTGCGATGCAGAGTC
TGCTCGACAGAT (Seq. ID No. 15)

35

mda-18: Gene which displays increased expression to IFN- β

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+ MEZ treated H0-1 cells after 24 hours (HP 2-45).
(Identical to hnRNP A1 protein (Gb-Pr:Humrnpa1), RNA
binding protein (Gb-Pr:Humhnrnpa)).

5 TACGATCAGACTGTTACATTTAGCAATCAACAGCATGGGGCGAAAAAAAAAAAAATC
TACTTAAAACCCTTTGTTGGAATGCTTTTACACTTTCCACAGAACAGAACTAAAA
TAACTGTTTACATTAGTCACAATACAGTCTCGA (Seq. ID No. 16)

Fifth Series of Experiments

5 The carcinogenic process often proceeds through a series
of interrelated stages and is regulated by multiple
genetic changes and environmental factors (1-6).
Although the specific events controlling each component
of this multistep process remain to be defined, a
recurrent theme in many cancer cells is an aberrant
pattern of differentiation (7-10). In addition, as
10 cancer cells evolve, ultimately developing new phenotypes
or an increased expression of pre-existing
transformation-related phenotypes, the degree of
expression of differentiation associated traits is often
further diminished. Malignant melanoma epitomizes the
15 process of tumor progression and emphasizes the selective
nature of the metastatic phenotype and the growth
dominant properties of metastatic cells (11-14). Of the
numerous types of cancer developing in North American
populations, melanoma is increasing at the fastest rate
20 and it is estimated that as many as 1 in 100 currently
born children may eventually develop superficial
spreading type melanoma (11). Although melanoma is
readily curable at early stages, surgical and
chemotherapeutic interventions are virtually ineffective
25 in preventing metastatic disease and death in patients
with advanced stages of malignant melanoma. These
observations emphasize the need for improved therapeutic
approaches to more efficaciously treat patients with
metastatic melanoma.

30

Development of malignant melanoma in humans, with the
exception of nodular type melanoma, consists of a series
of sequential alterations in the evolving tumor cells
(11-15). These include conversion of a normal melanocyte
35 into a common acquired melanocytic nevus (mole),

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followed by the development of a dysplastic nevus, a radial growth phase (RGP) primary melanoma, a vertical growth phase (VGP) primary melanoma and ultimately a metastatic melanoma (Figure 20). Although readily

5 treatable during the early stages of development, even during the VGP if the lesion is $\leq$ 0.76-mm thick, currently employed techniques are not very effective (<20% survival) in preventing metastatic spread and morbidity in patients with VGP lesions > 4.0-mm thickness

10 (11). This exceptional model system is ideally suited to evaluate the critical gene expression changes that mediate both the early and late phases of melanoma evolution.

15 A potentially less toxic approach to cancer therapy involves a process termed differentiation therapy (7,9,10,16,17). Two premises underlie this therapeutic modality. (A) Many types of neoplastic cells display aberrant patterns of differentiation resulting in

20 unrestrained growth; and (B) Treatment with the appropriate agent(s) can result in the reprogramming of tumor cells to lose proliferative capacity and become terminally differentiated. Intrinsic in this hypothesis is the assumption that the genes that mediate normal

25 differentiation in many tumor cells are not genetically defective, but rather they fail to be expressed appropriately. The successful application of differentiation therapy in specific instances may result because the appropriate genes inducing the differentiated

30 phenotype become transcriptionally activated resulting in the production of necessary gene products required to induce terminal cell differentiation. Applicants have tested this hypothesis using human melanoma cells (8,10,18-23). Treatment of human melanoma cells with the

35 combination of recombinant human fibroblast interferon

(IFN- β) and the antileukemic compound mezerein (MEZ) results in a rapid cessation of growth, an induction of morphological changes, an alteration in antigenic phenotype, an increase in melanin synthesis and an irreversible loss in proliferative capacity, i.e., terminal cell differentiation (18,21,23). In contrast, treatment of the same melanoma cells with equivalent doses of either IFN- β or MEZ alone results in specific differentiation-related and immunologically-related changes and growth suppression, but terminal differentiation does not occur (18,21,24-34).

This invention summarizes the results of applicants' analysis of the process of reversible and irreversible (terminal) differentiation in human melanoma cells. By using the technique of subtraction hybridization a series of novel genes, termed melanoma differentiation associated (*mda*), have been identified that display enhanced expression during differentiation and growth arrest in human melanoma cells. These newly identified *mda* genes should prove useful in defining the molecular basis of human melanoma growth, differentiation and transformation progression.

Dissecting the Processes of Growth Control and Differentiation in Human Melanoma Cells

The process of terminal differentiation in H0-1 cells involves a number of changes in cellular phenotype and gene expression (18,21,23,35). Biochemical and cellular changes include growth suppression, changes in melanin synthesis (biochemical differentiation) and modified antigenic properties (immunologic differentiation) (18,21,23,29,34,35). The ability to define the relationship between the different components of growth

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and differentiation and the corresponding gene expression changes induced in human melanoma cells has been assisted by the identification of specific compounds that induce different components of these processes (Figure 21A-H)
5 (18,21,23,33,36).

Growth of H0-1 cells in the combination of IFN- β plus recombinant immune interferon (IFN- γ) results in a similar level of growth suppression after 96 hr as does
10 the combination of IFN- β + MEZ (23,33). However, this combination of interferons induces reversible growth arrest and it does not cause an increase in melanin synthesis above that induced by IFN- β alone (33). This specific combination of agents permits a dissociation
15 between reversible growth suppression and induction of irreversible growth suppression and terminal differentiation. Treatment of H0-1 cells with compounds such as all-trans retinoic acid (RA), mycophenolic acid (MPA), IFN- β and MEZ results in a reversible increase in
20 melanin synthesis, i.e., reversible biochemical differentiation (Figure 21A-H) (23,36). However, although MPA, IFN- β and MEZ induce growth suppression, RA increases melanin and tyrosinase levels without altering H0-1 growth (23,26]. These results indicate that RA can
25 be used to identify gene expression changes correlating directly with increased melanin synthesis in the absence of growth suppression. Additional compounds that have proven of interest, include MPA and MEZ that induce morphologic differentiation in H0-1 cells (Figure 22).
30 These changes in H0-1 cells are also reversible following growth in the absence of the inducing agent. The only currently available combination of agents that can irreversibly induce the spectrum of differentiation changes resulting in terminal cell differentiation in H0-1
35 cells is IFN- β + MEZ (18,21,23).

Table 3. Gene Expression Changes Observed in Terminally Differentiated H0-1 Cells

	<u>Increased</u> <u>Expression</u>	<u>Decreased</u> <u>Expression</u>	<u>No</u> <u>Change in</u> <u>Expression</u>
5			
	c-jun	c-myc	c-fos
10	jun-B	cyclin A	RB
	HLA Class I	cyclin B	N-cadherin
15	ISG-15	tenascin	
	ISG-54	γ -actin	
	gro/MGSA	β -actin	
20	α_5 integrin	cdc 2	
	β_1 integrin	histone H1	
25	fibronectin	histone H4	
30	H0-1 cells were grown for 96 hr in the presence of 2000 units/ml IFN- β and 10 ng/ml MEZ prior to isolation of total RNA and analysis of gene expression by Northern blotting analysis (23). Cells remain viable under these conditions, but they irreversibly lose proliferative ability (18,23).		

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Autocrine Factors Involved in Gene Expression Changes
Induced in Human Melanoma Cells During Reversible Growth
Suppression and Terminal Cell Differentiation

5

A potentially important mediator of growth arrest during differentiation in hematopoietic cells is autocrine IFN- β (37-39). Evidence suggesting a link between autocrine IFN- β and differentiation in hematopoietic cells include:

10 (a) the observation that IFN- β neutralizing antibodies can partially abrogate the reduction in c-myc levels and growth suppression occurring during hematopoietic differentiation; (b) the induction of interferon regulatory factor 1 (IRF-1) during myeloid

15 differentiation; (c) the partial reversal by IRF-1 antisense oligomers of growth inhibition and the induction of differentiation induced in leukemic cells by interleukin-6 and leukemia inhibitory factor; and (d) the induction of specific type I interferon (IFN- α/β) gene

20 expression during terminal differentiation in hematopoietic cells (37-39).

Enhanced expression of interferon responsive-genes and the gro/MGSA gene occurs in H0-1 cells during the

25 processes of reversible and irreversible differentiation (23). These observations suggest that autocrine-feedback pathways could contribute to the gene expression changes observed during the differentiation process. To directly test for this possibility, H0-1 cells were treated with

30 various inducing agents for 24 hr, cultures were washed free of inducers and then grown for 72 hr in medium lacking the inducing compounds. The conditioned medium from these cells was collected and tested for its ability to induce gene expression changes in naive H0-1 cells

35 (23). Conditioned medium obtained from cells treated

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with IFN- β + MEZ induced growth suppression and a number of gene expression changes also apparent in inducer-treated H0-1 cells (23,40). These include, enhanced c-jun, α , integrin and fibronectin expression and induction of jun-B, HLA Class I, ISG-15, and gro/MGSA expression (23). These observations suggest two autocrine loops may be associated with differentiation in H0-1 cells, one involving an autocrine IFN- β and the other an autocrine gro/MGSA. Support for the IFN- β autocrine loop is indicated by the ability of IFN- β antibodies to partially neutralize ISG-15 induction by conditioned medium and the direct induction of the IFN- β gene as monitored by RT-PCR by conditioned medium (40). However, conditioned medium from IFN- β + MEZ-treated H0-1 cells does not induce terminal differentiation in H0-1 cells (40). Similarly, additional agents that induce reversible growth arrest and differentiation also produce conditioned medium that can induce type I interferon responsive genes in naive H0-1 cells. These results suggest that specific autocrine loops may also contribute to growth inhibition and the differentiation process in solid tumors such as human melanoma.

Identification of Genes Differentially Expressed During the Processes of Differentiation and Growth Suppression in Human Melanoma Cells

To directly identify genes displaying differential expression in human melanoma cells induced to terminally differentiate applicants have used a modified subtraction hybridization approach (Figure 8) (41). cDNA libraries were prepared from poly (A+) RNA obtained from untreated H0-1 cells (Ind cDNA library; driver cDNA library), and H0-1 cells treated with IFN- β + MEZ for 2, 4 8, 12 and 24

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(Ind⁻ cDNA library; tester cDNA library). Tester and driver cDNA libraries were directionally cloned into the commercially available λ Uni-ZAP phage vector. Subtraction hybridization was then performed between double-stranded tester DNA and single-stranded driver DNA prepared by mass excision of the libraries. The subtracted cDNAs were efficiently cloned into the λ Uni-ZAP phage vector, which permits easy manipulation for both screening and gene characterization. A single round of subtraction of untreated H0-1 control (Ind⁻) cDNAs from IFN- β + MEZ-treated (Ind⁺) cDNAs resulted in the identification of a series of cDNAs displaying differential expression in untreated versus differentiation inducer-treated H0-1 cells. These cDNAs are referred to as melanoma differentiation associated (mda) cDNAs. Initially 70 cDNA clones were analyzed and 23 clones were found to display differences in gene expression between Ind⁻ and Ind⁺-treated H0-1 cells (41). As anticipated, subtraction of control H0-1 cDNAs from IFN- β + MEZ-treated H0-1 cDNAs resulted in a series of MDA genes that displayed enhanced expression after 24-h treatment with the various inducers. These included genes displaying enhanced expression in H0-1 cells treated with both IFN- β and IFN- β + MEZ (mda-1 and mda-2), MEZ and IFN- β + MEZ (mda-3), IFN- β , MEZ and IFN- β + MEZ (mda-4) and uniquely by IFN- β + MEZ (mda-5 and mda-6) (Figure 10) (41). Of these six mda genes, only mda-3 originally represented a previously identified gene, GOS-19-1 (41). Analysis of eight additional human melanoma cell lines indicates that specific mda genes also display enhanced expression following a 24-h treatment with IFN- β + MEZ (data not shown).

The studies described above indicate that specific mda

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genes exhibit elevated expression in H0-1 cells after 24-h treatment with appropriate inducing agents. Studies were performed to monitor *mda* expression in H0-1 cells treated with IFN- β , MEZ or IFN- β + MEZ for 96-h (Figure 15). Additionally, the pattern of expression of specific *mda* genes under experimental conditions inducing reversible growth suppression (IFN- β + IFN- γ , IFN- β , IFN- γ , MEZ, MPA, MPA + MEZ, RA + MEZ), increased melanin synthesis (IFN- β , MEZ, MPA, RA, IFN- β + MEZ, MPA + MEZ, RA + MEZ), increased melanin synthesis (IFN- β , MEZ, MPA, RA, IFN- β + MEZ, MPA + MEZ, RA + MEZ), morphological changes (MPA, MEZ, IFN- β + MEZ, MPA + MEZ, RA + MEZ) or terminal cell differentiation (IFN- β + MEZ) was determined. These experiments indicate continuous elevated expression of *mda*-4, *mda*-5, *mda*-6 (p21), *mda*-7, *mda*-8 and *mda*-9 in terminally differentiated H0-1 cells (Figure 15). A differential pattern of expression of the *mda* genes was observed in H0-1 cells treated with the various differentiation and growth modulating agents. Three of the *mda* cDNAs, *mda*-4, *mda*-5 and *mda*-8, displayed overlapping induction profiles in H0-1 cells. These genes displayed elevated expression in H0-1 cells treated for 96 hr with IFN- β + MEZ, IFN- γ or IFN- β + IFN- γ (Figure 15). These cDNAs, which have not been previously described in any DNA data base, may correspond to new classes of cytokine-responsive genes. This possibility is currently under investigation. Expression of *mda*-7 was increased in H0-1 cells treated for 96 h with agents inducing growth arrest, including IFN- β , MEZ, MPA, IFN- β + IFN- γ , IFN- β + MEZ, MPA + MEZ, RA + MEZ. The degree of increase in *mda*-7 expression was greatest in H0-1 cells treated with IFN- β + MEZ that also induces terminal cell differentiation. Treatment of H0-1 cells for 96 hr with RA does not induce growth changes or induce *mda*-7 expression. Similarly, IFN- γ that only marginally inhibits H0-1

DEMANDES OU BREVETS VOLUMINEUX

**LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVETS
COMPREND PLUS D'UN TOME.**

CECI EST LE TOME __1__ DE __2__

NOTE: Pour les tomes additionels, veuillez contacter le Bureau Canadien des Brevets.

JUMBO APPLICATIONS / PATENTS

**THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE
THAN ONE VOLUME.**

THIS IS VOLUME __1__ OF __2__

NOTE: For additional volumes please contact the Canadian Patent Office.

What is claimed is:

1. An isolated *mda-7* nucleic acid encoding the MDA-7 amino acid sequence of SEQ ID NO:7.
2. The nucleic acid of claim 1 further comprising a promoter wherein the promoter controls the expression of the nucleic acid.
3. The nucleic acid of claim 2, wherein the promoter is an inducible promoter.
4. The nucleic acid of claim 2, wherein the promoter is a tissue-specific promoter.
5. The nucleic acid of claim 2, wherein the promoter is a bacteriophage promoter.
6. The nucleic acid of claim 5, wherein the bacteriophage promoter is T3, T7 or SP6.
7. The nucleic acid of claim 2, wherein the promoter is the promoter of an RNA transcriptase.
8. A pharmaceutical composition which comprises the nucleic acid of any one of claims 1 to 7 and a pharmaceutically acceptable carrier.
9. The pharmaceutical composition of claim 8, wherein the nucleic acid is combined with a liposome.
10. A vector comprising the nucleic acid of claim 1.
11. The vector of claim 10, wherein the vector is a plasmid, a bacteriophage, a cosmid or a retroviral vector.
12. A host cell comprising the vector of claim 10.
13. A method of detecting the expression of the *mda-7* gene in a cell comprising:
a) isolating the nucleic acids in the cell; b) hybridizing the isolated nucleic acids with a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with the *mda-7* cDNA nucleotide sequence of SEQ ID NO:6 under conditions

permitting formation of hybrids; and c) detecting hybrids formed, the detection of the hybrids formed indicating the expression of the *mda-7* gene in the cell.

14. An isolated protein comprising the amino acid of SEQ ID NO:7.
15. An isolated protein comprising a protein encoded by a nucleic acid having the sequence of SEQ ID NO:6.
16. An isolated protein comprising the sequence of SEQ ID NO:7 produced by a host cell into which a nucleic acid, comprising the sequence of SEQ ID NO:6, operably linked to a promoter element, has been introduced.
17. An antibody that specifically binds to a MDA-7 protein having a sequence of SEQ ID NO:7.
18. Use of the antibody of claim 17 to detect expression of MDA-7 protein having a sequence of SEQ ID NO:7.

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FIGURE 1A

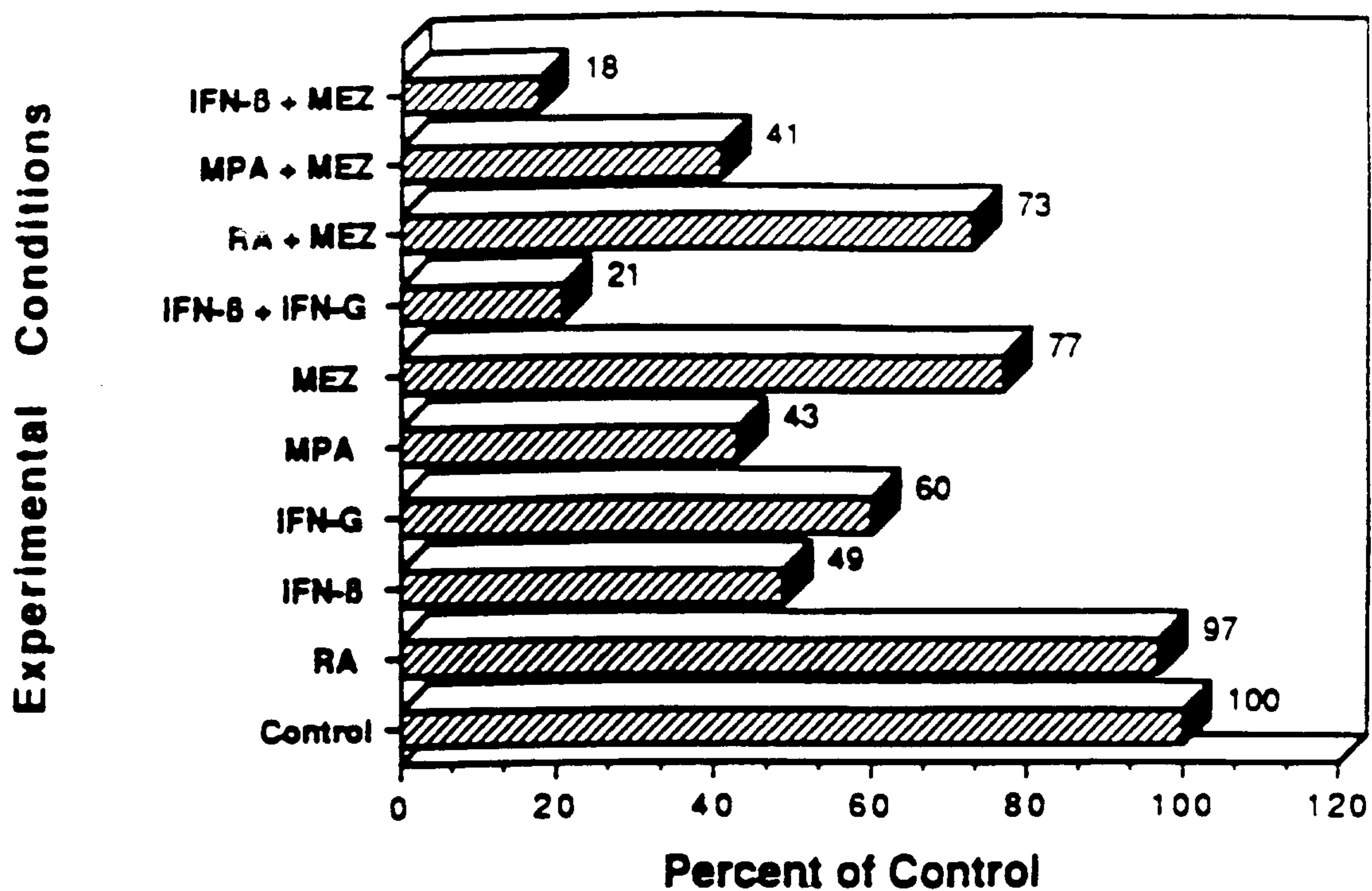
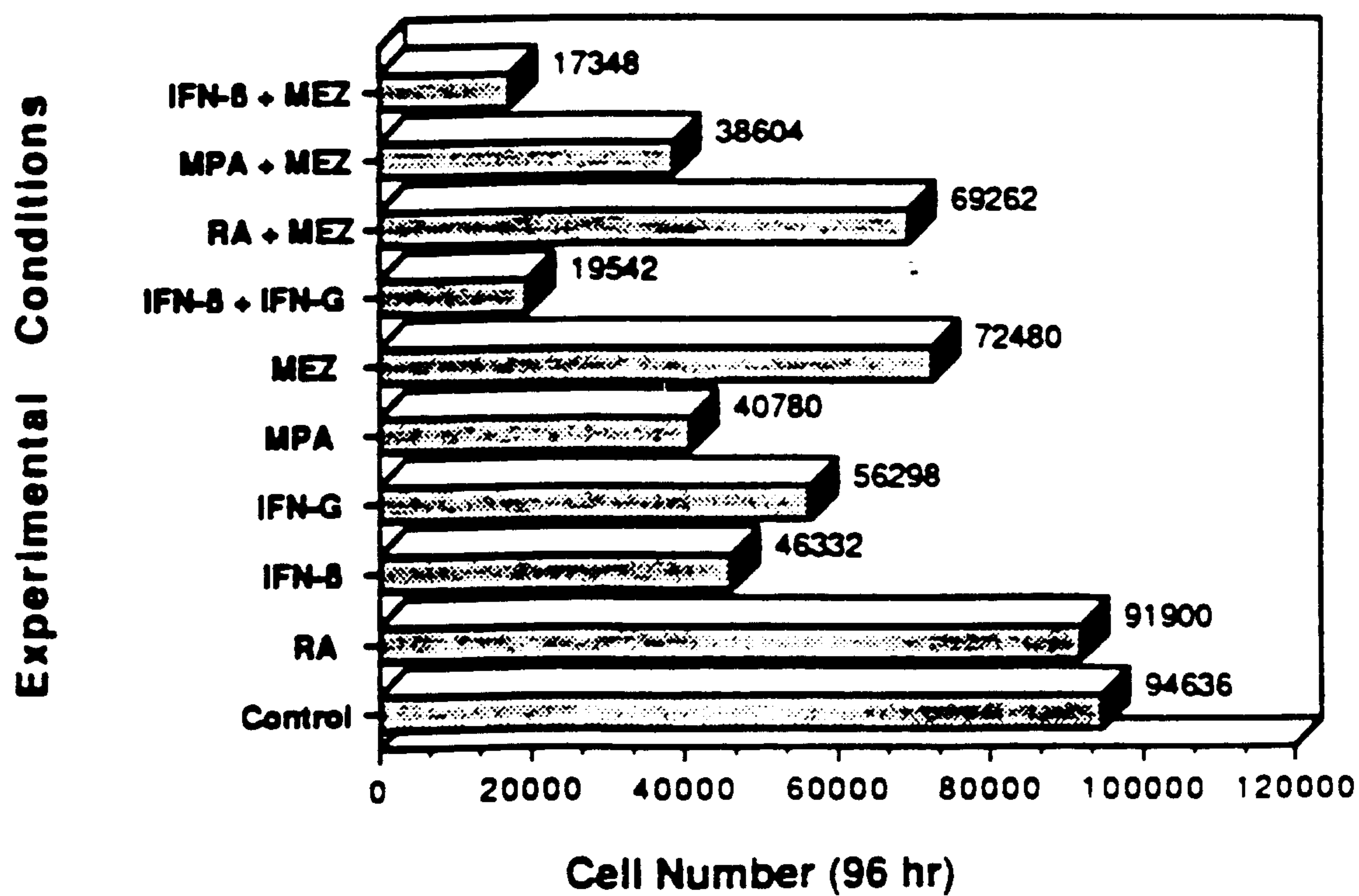
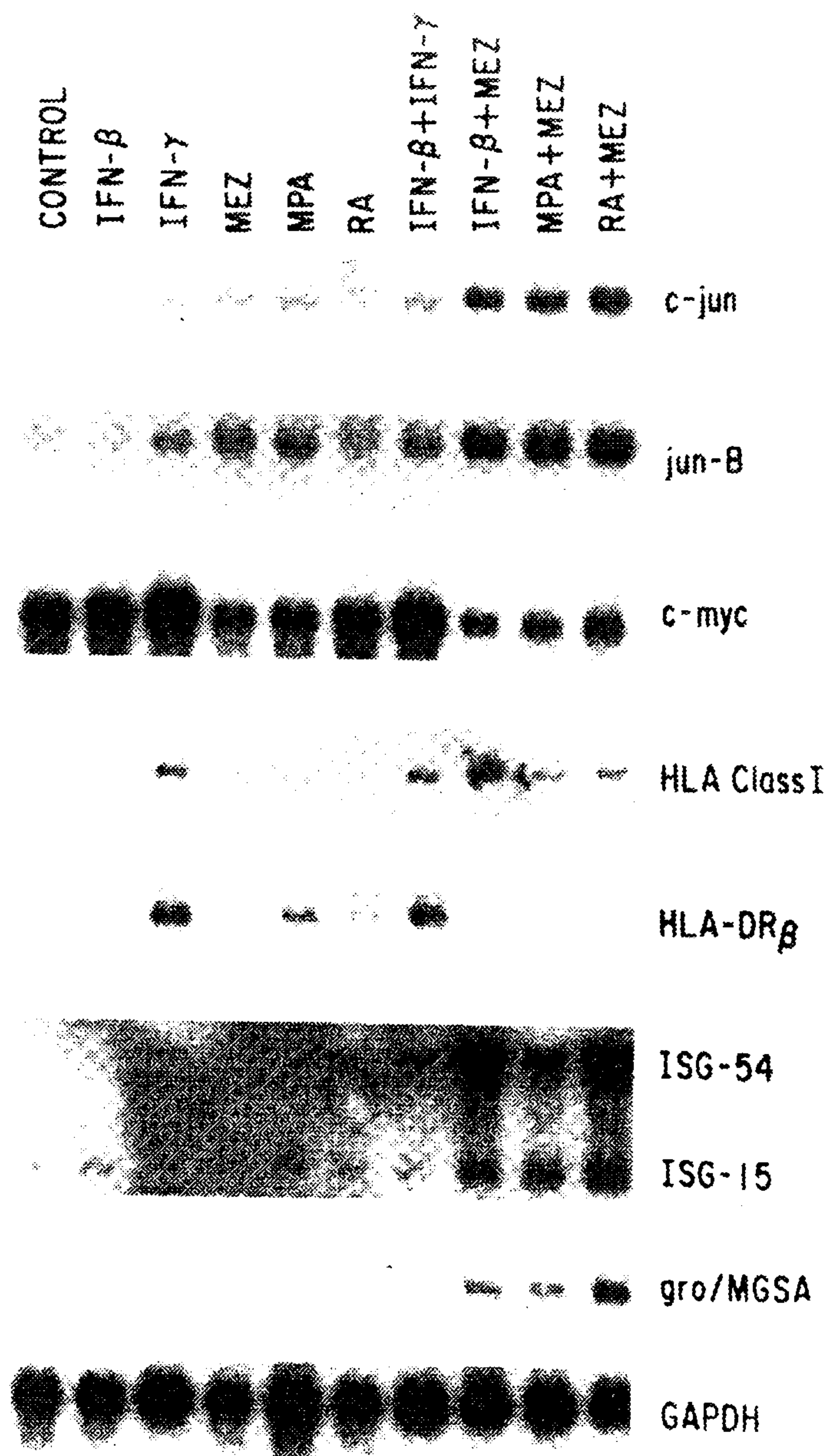


FIGURE 1B



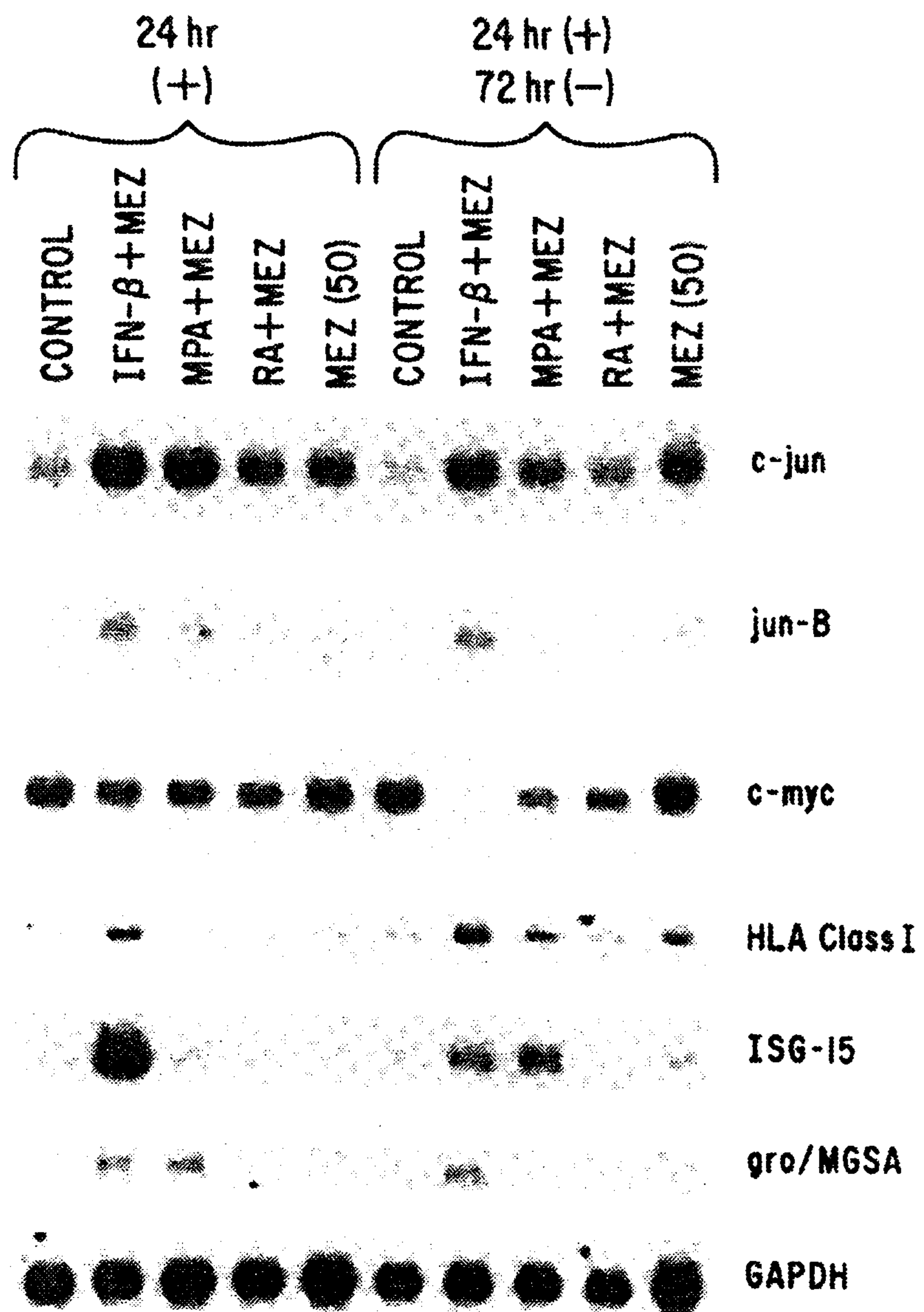
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FIGURE 2



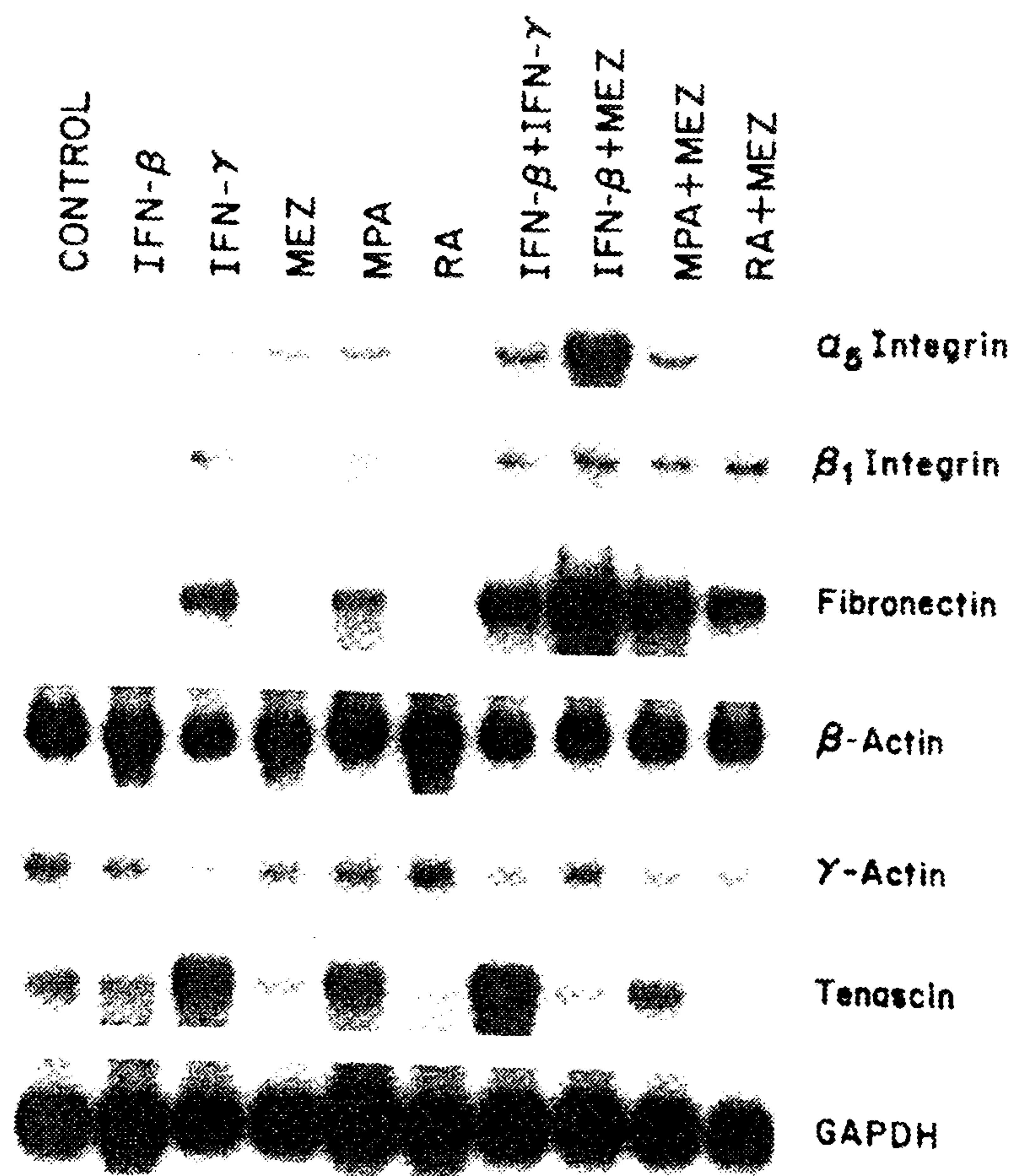
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FIGURE 3



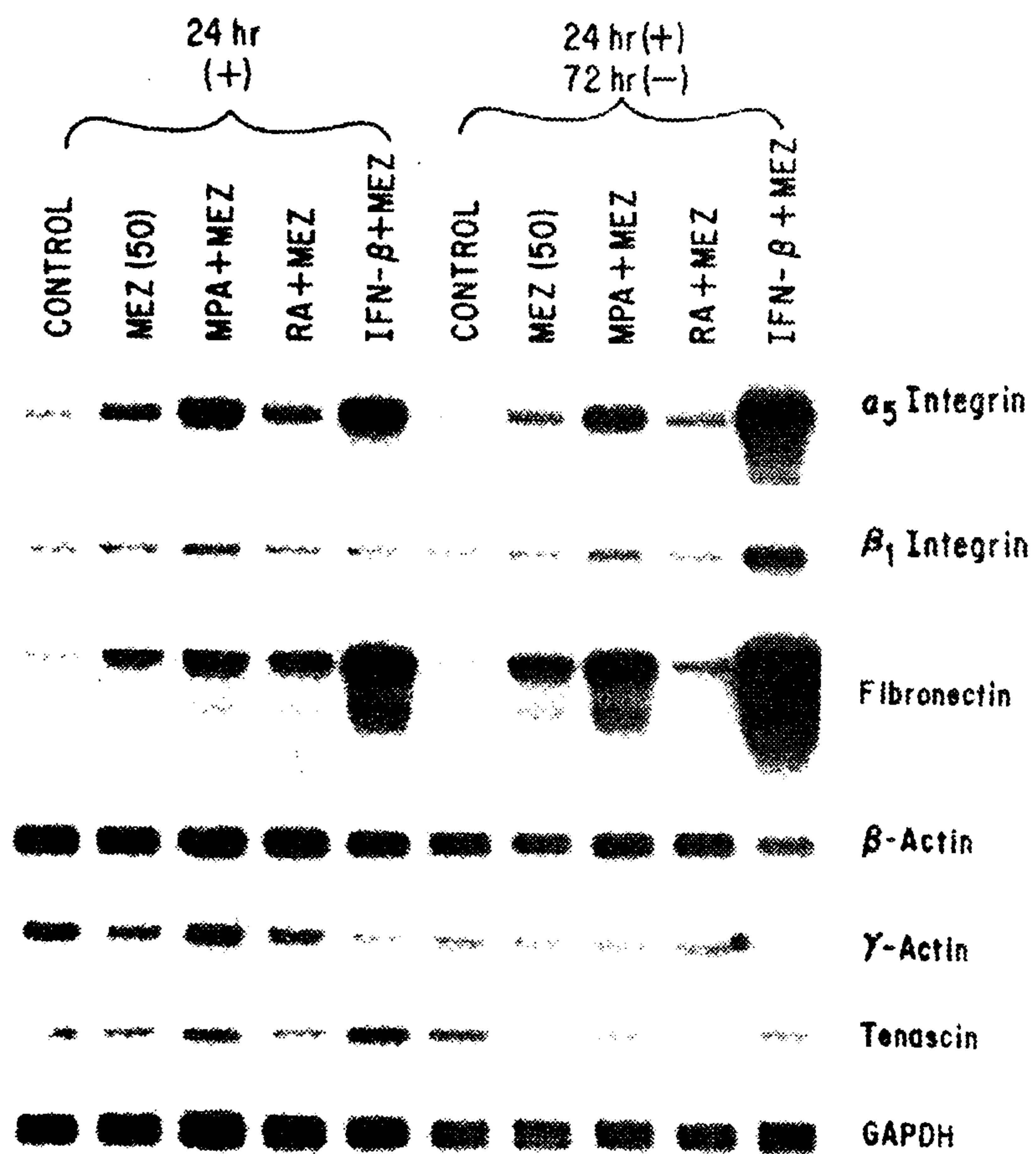
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FIGURE 4



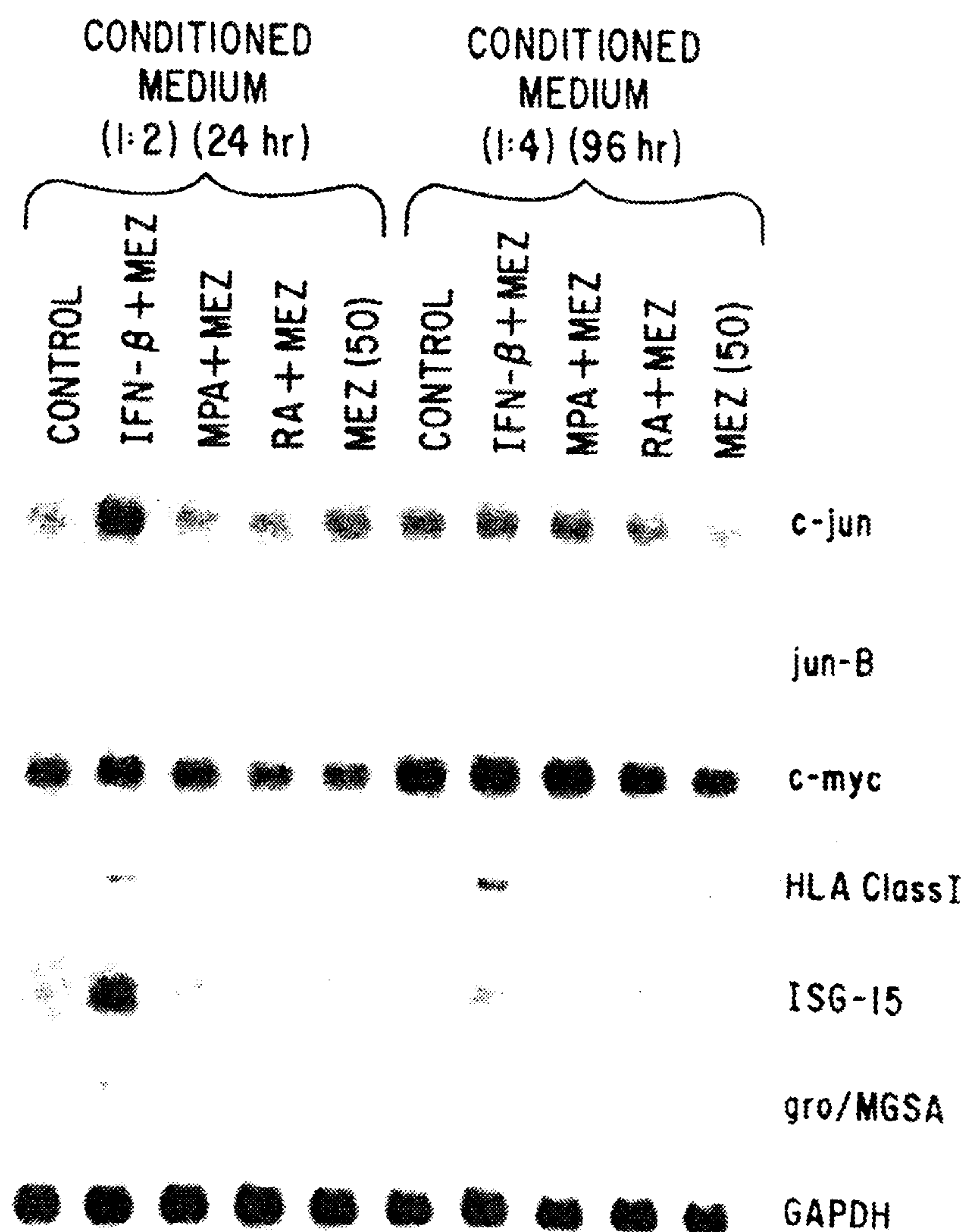
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FIGURE 5



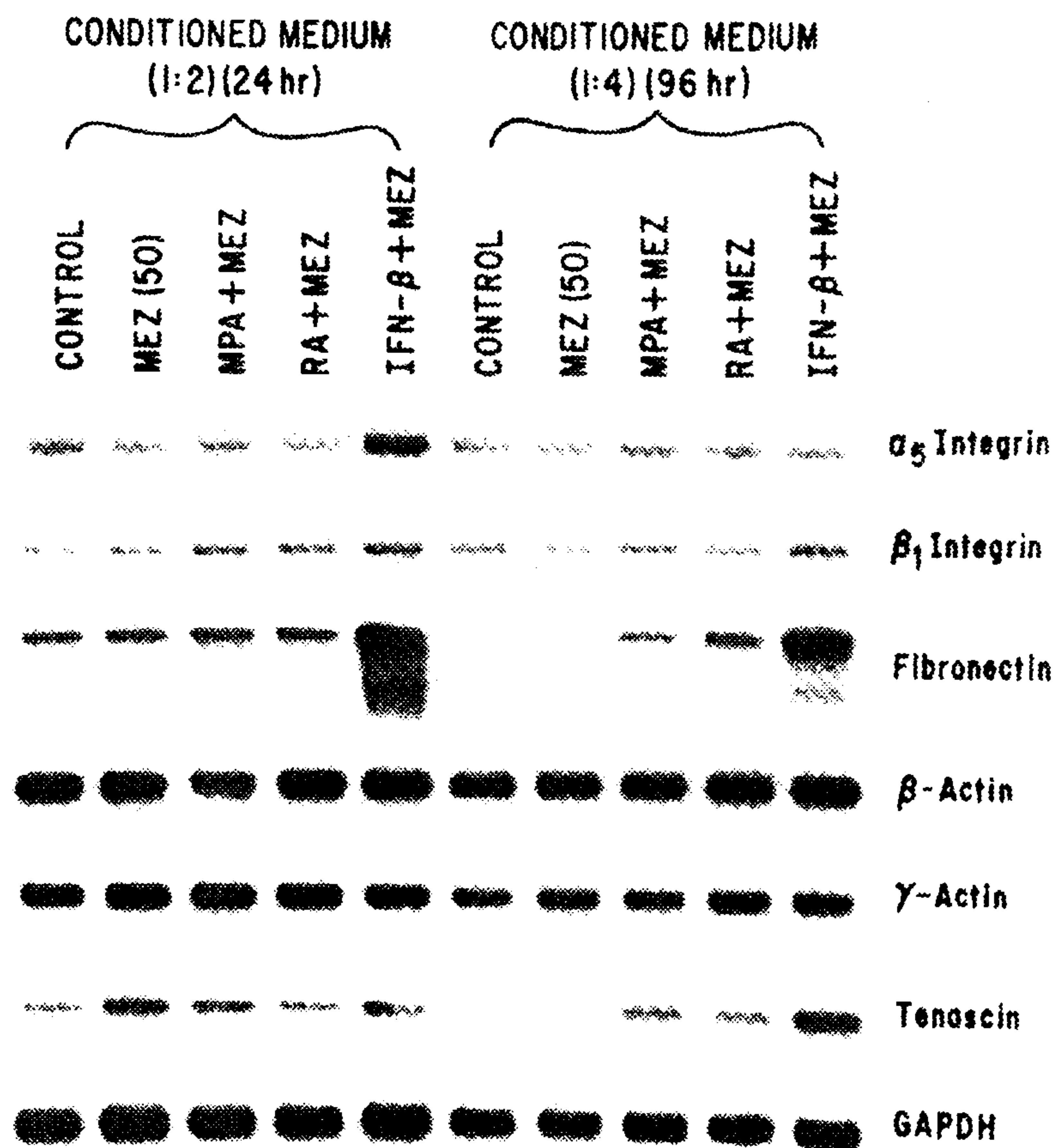
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FIGURE 6



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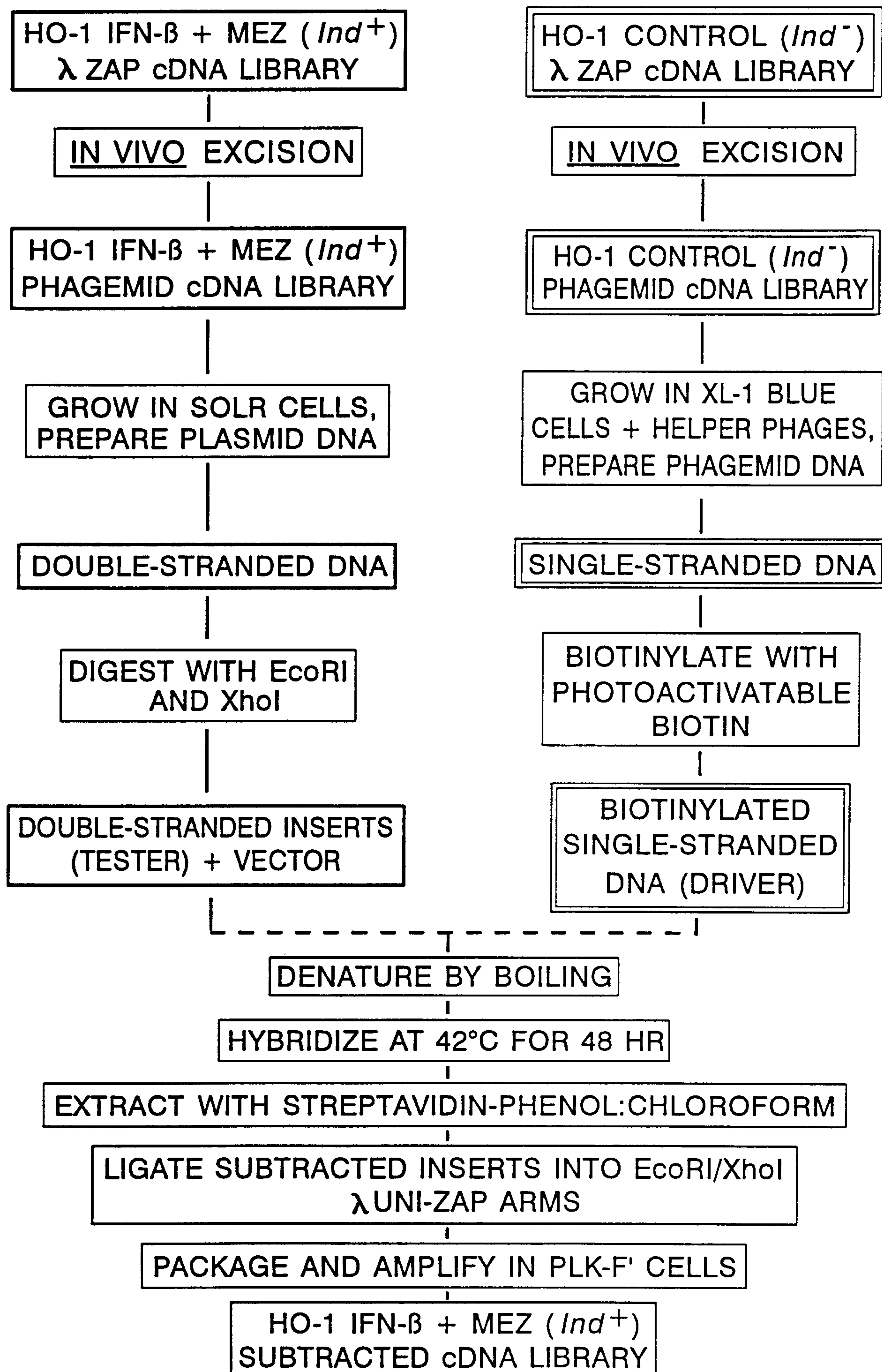
FIGURE 7



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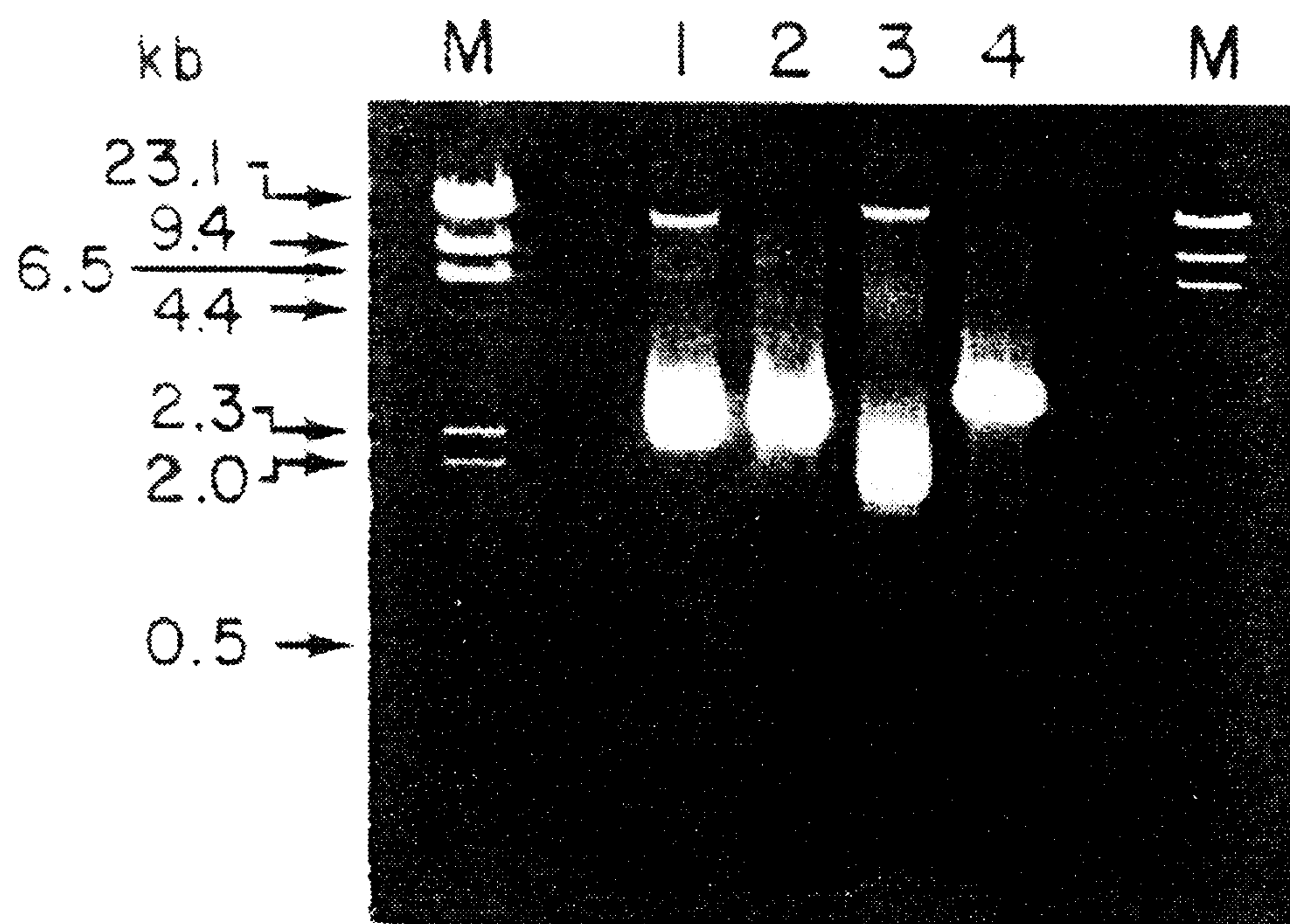
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FIGURE 8



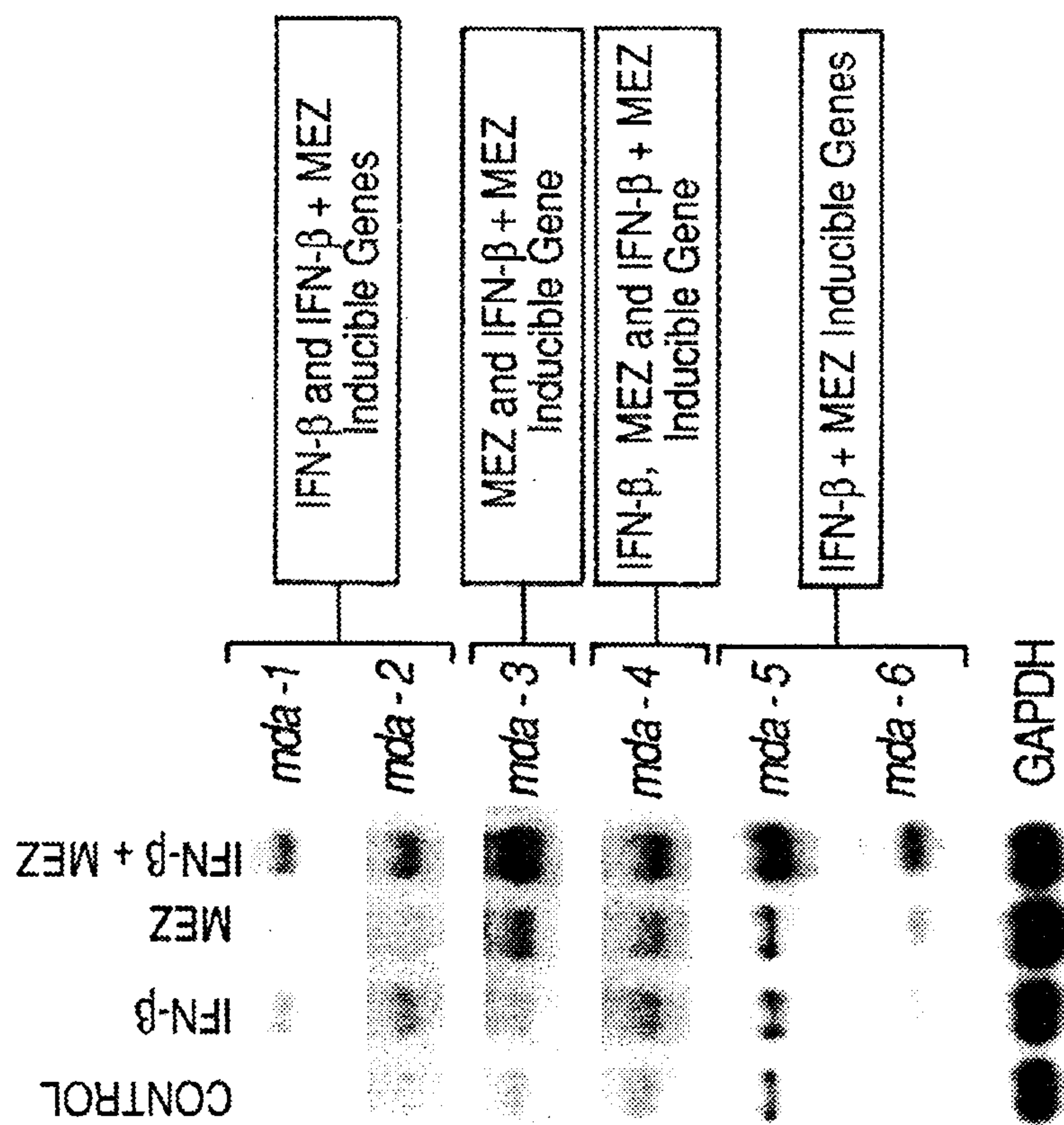
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FIGURE 9



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FIGURE 10



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FIGURE 11

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1  TCCTCCCTCTGCACCATGGCTCTCTSCAACCAAGTTCTCTGCAATCATTGCT  50
   | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
112 TCCTCCCTCTGCACCATGGCTCTCTGCAACCAAGTTCTCTGCAATCATTGCT  161

51  GCTGACACGCCGACCGCTGCTGCTTCAAGCTACACCTCCGBCAGATTCC  100
   | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
162 GCTGACACGCCGACCGCTGCTGCTTCAAGCTACACCTCCGBCAGATTCC  211

101 ACAGAAATTTCAATAGCTGACTACTTTGAGACGAGCAGCCAGTGCTCCAGC  150
   | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
212 ACAGAAATTTCAATAGCTGACTACTTTGAGACGAGCAGCCAGTGCTCCAGC  261

151 CCGGTGTCACTTCCTAACCAAGCGAACCAGGCAAGGCTCTGTGACCC  200
   | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
262 CCGGTGTCACTTCCTAACCAAGCGAACCAGGCAAGGCTCTGTGACCC  311

201 AGTGAGGAGTGGGTCCAGAAATATGTCAAGCAGCTGAGCTGAGT  245
   | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
312 AGTGAGGAGTGGGTCCAGAAATATGTCAAGCAGCTGAGCTGAGT  326

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FIGURE 12

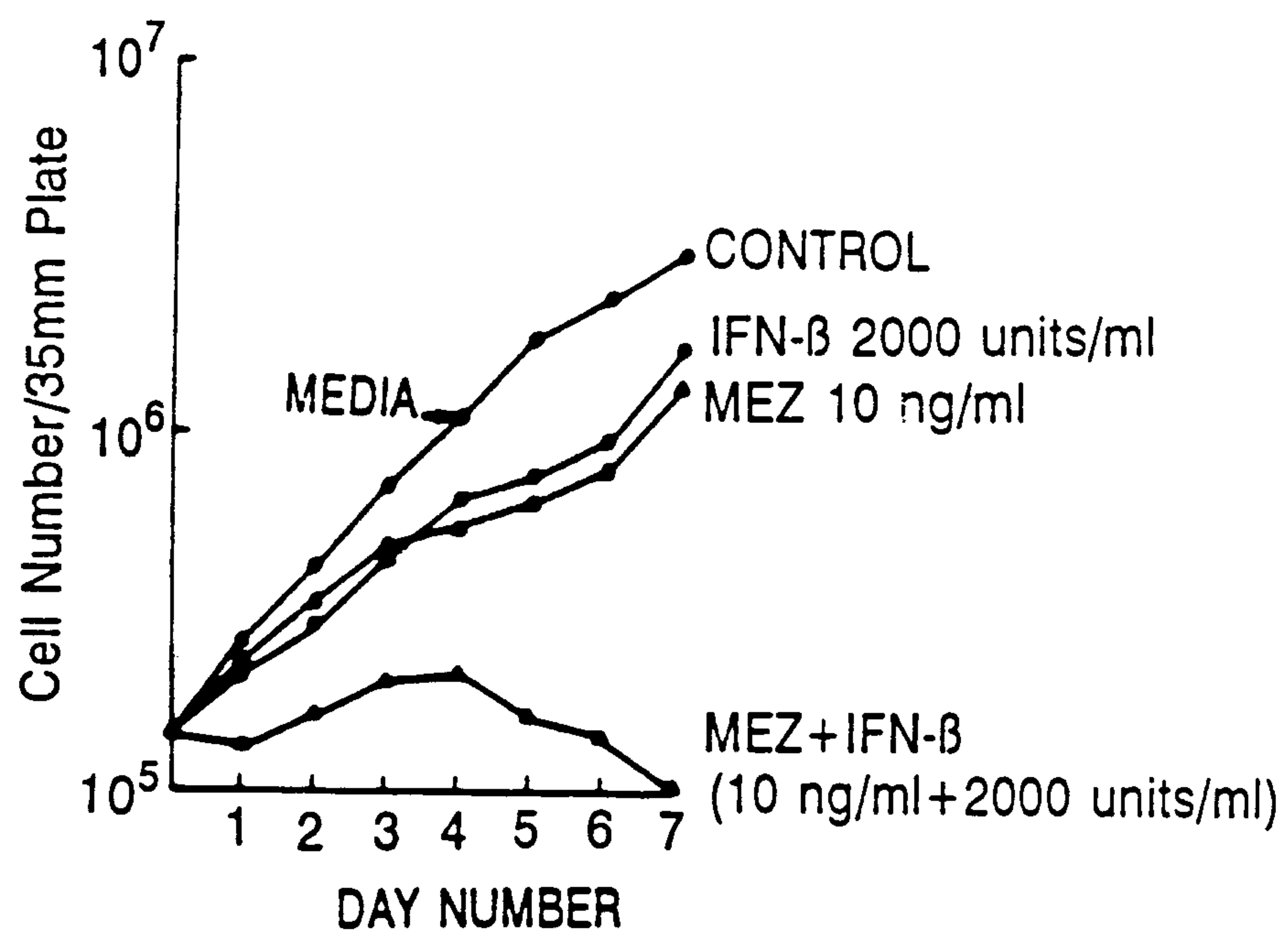
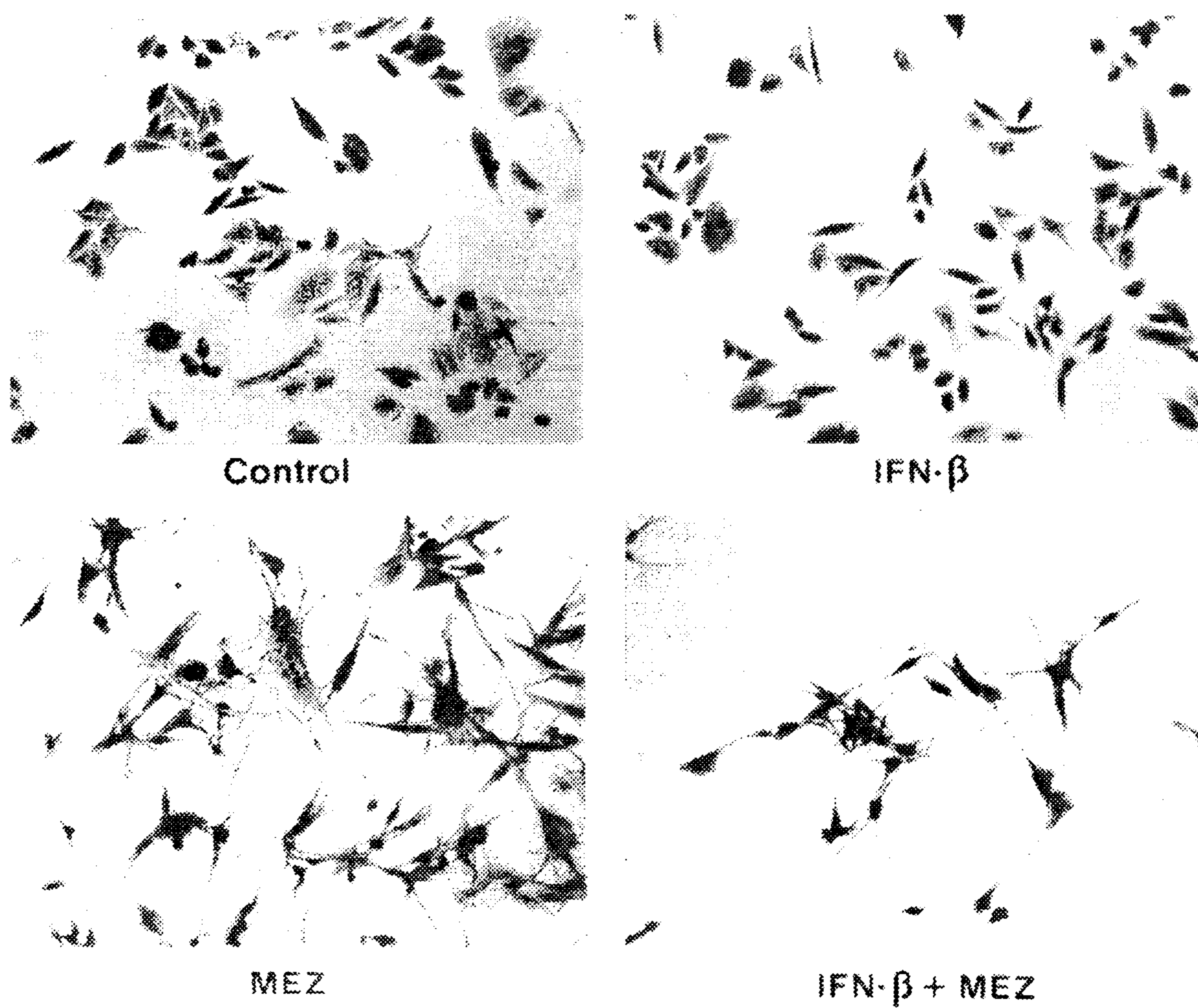


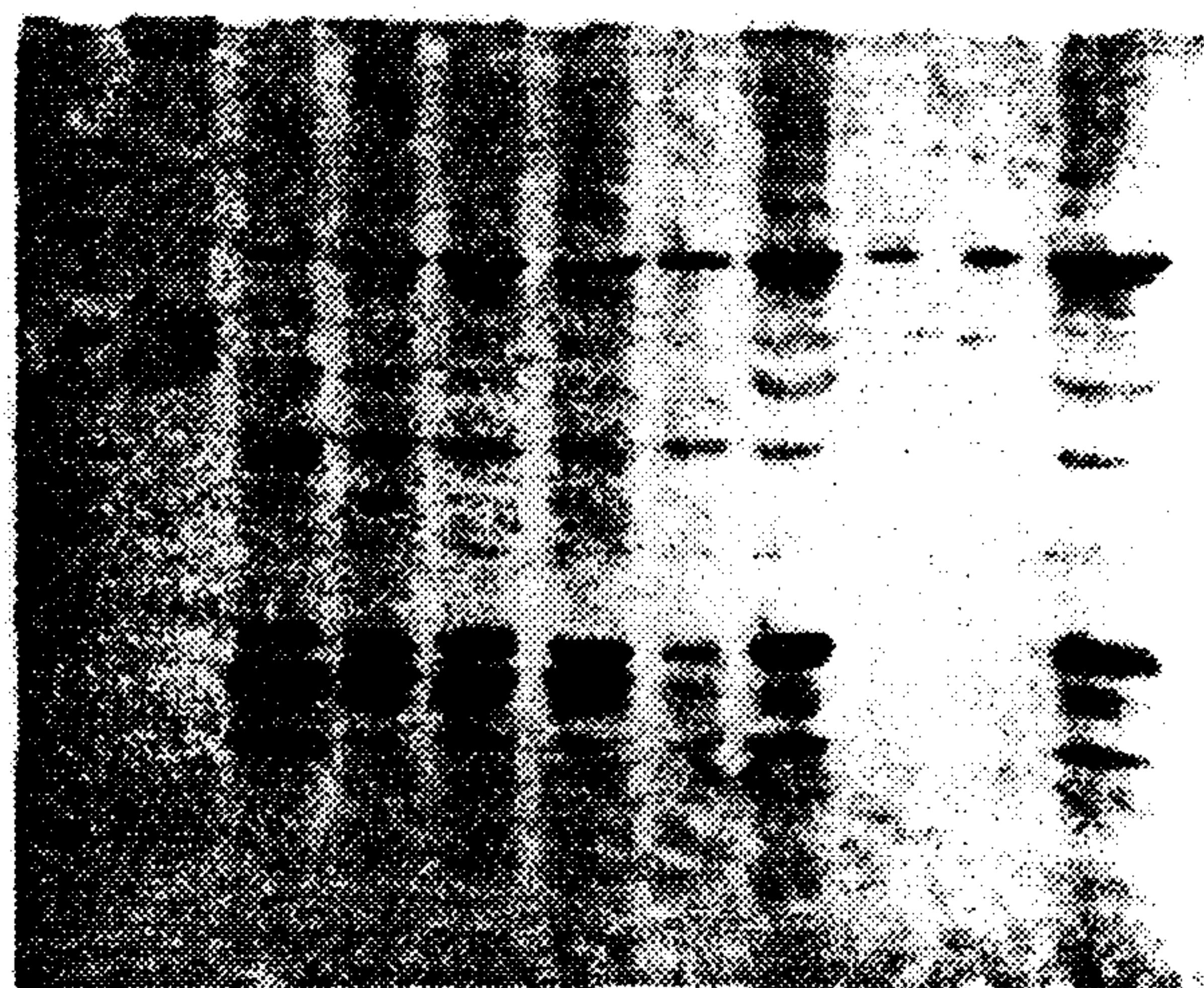
FIGURE 13



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FIGURE 14

MARKERS
3T3T
FM 517 CONTROL
FM 517 B1000 + MEZ10
FM 517 B1000 + MEZ 50
FM 517 MEZ10 + MPA 3
FO-1 CONTROL
FO-1 B250 + MEZ50
FO-1 MEZ10 + MPA 3
HO-1 CONTROL

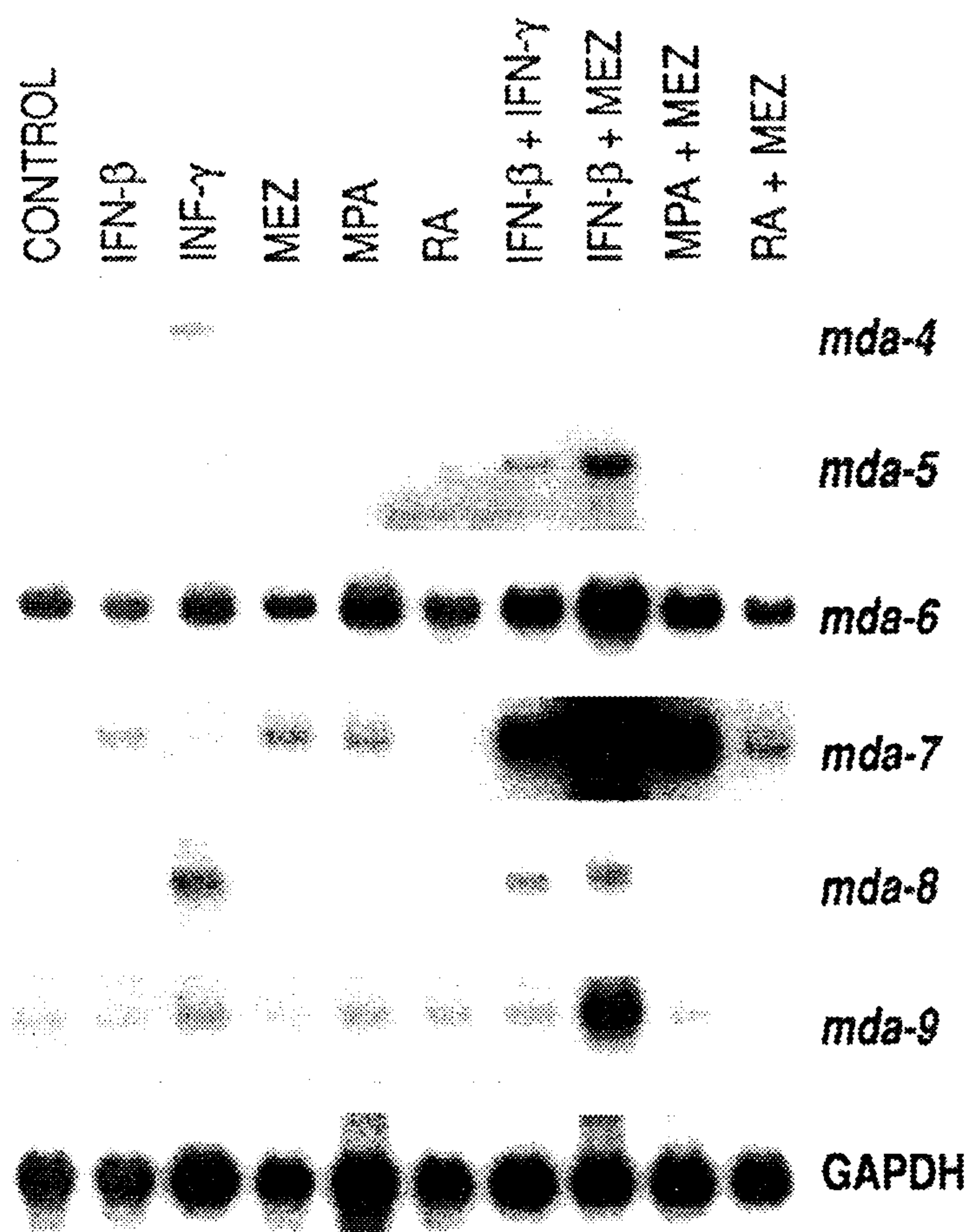


← hsp90

] P2Ps

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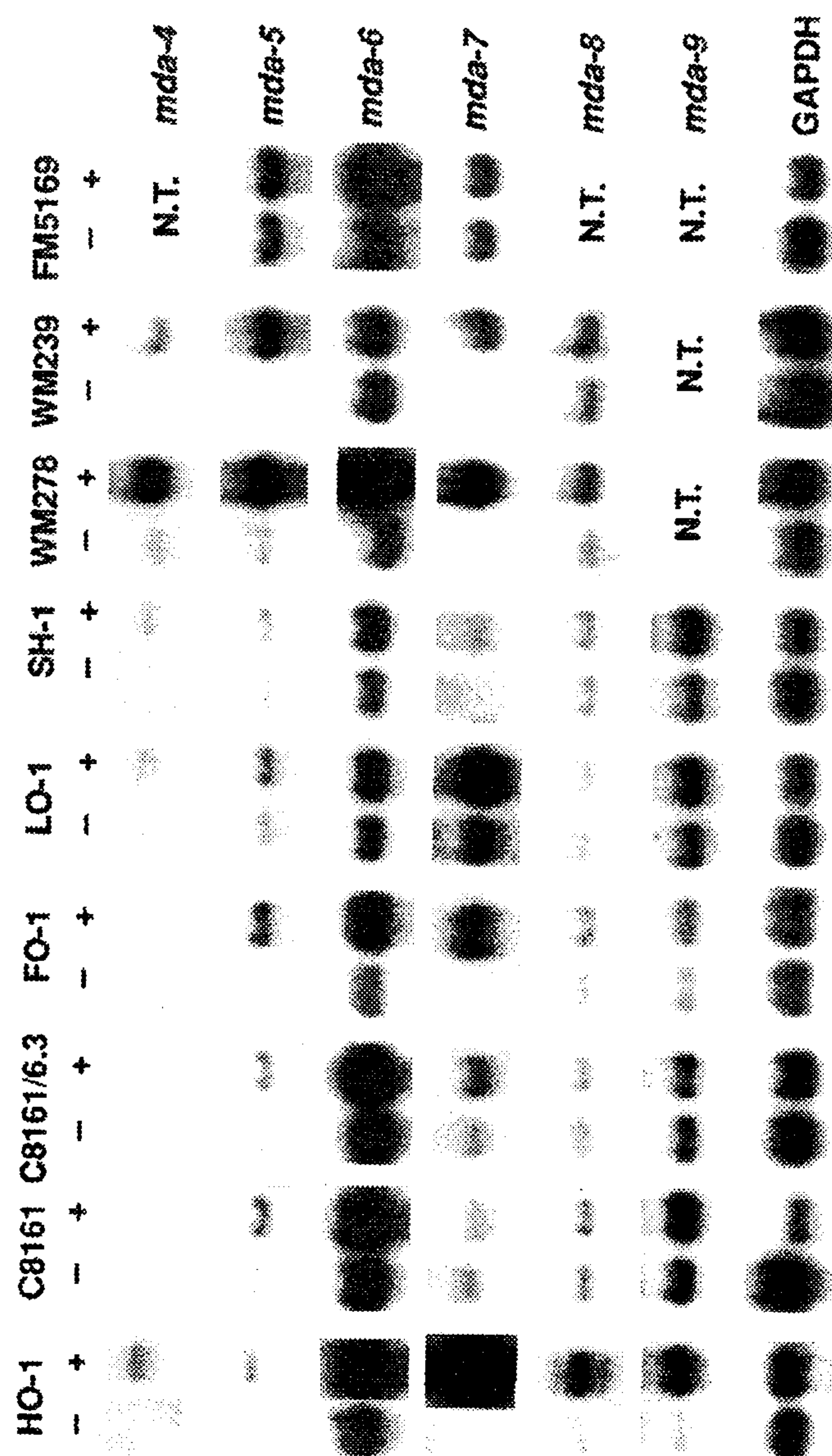
FIGURE 15



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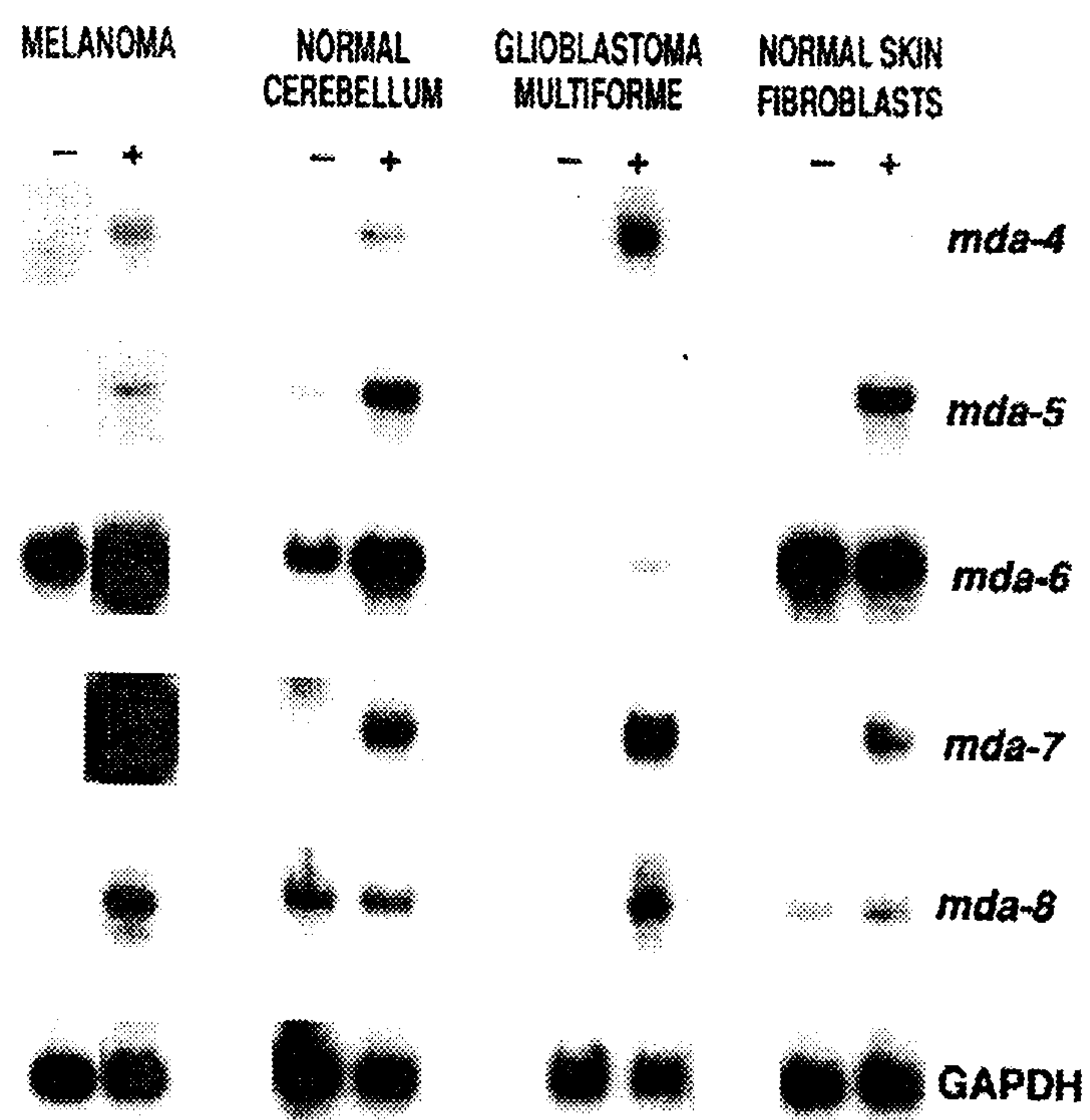
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FIGURE 16



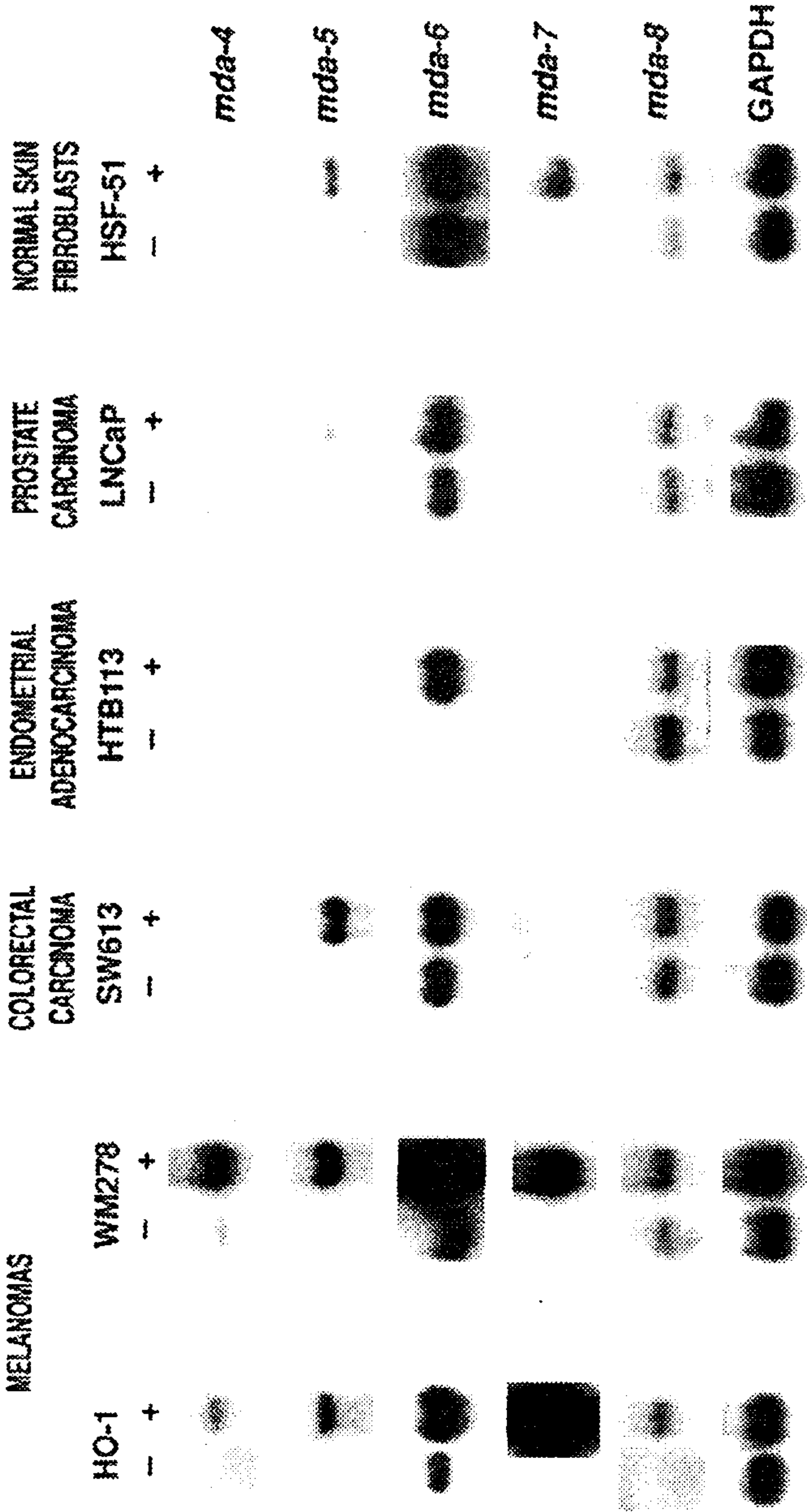
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FIGURE 17



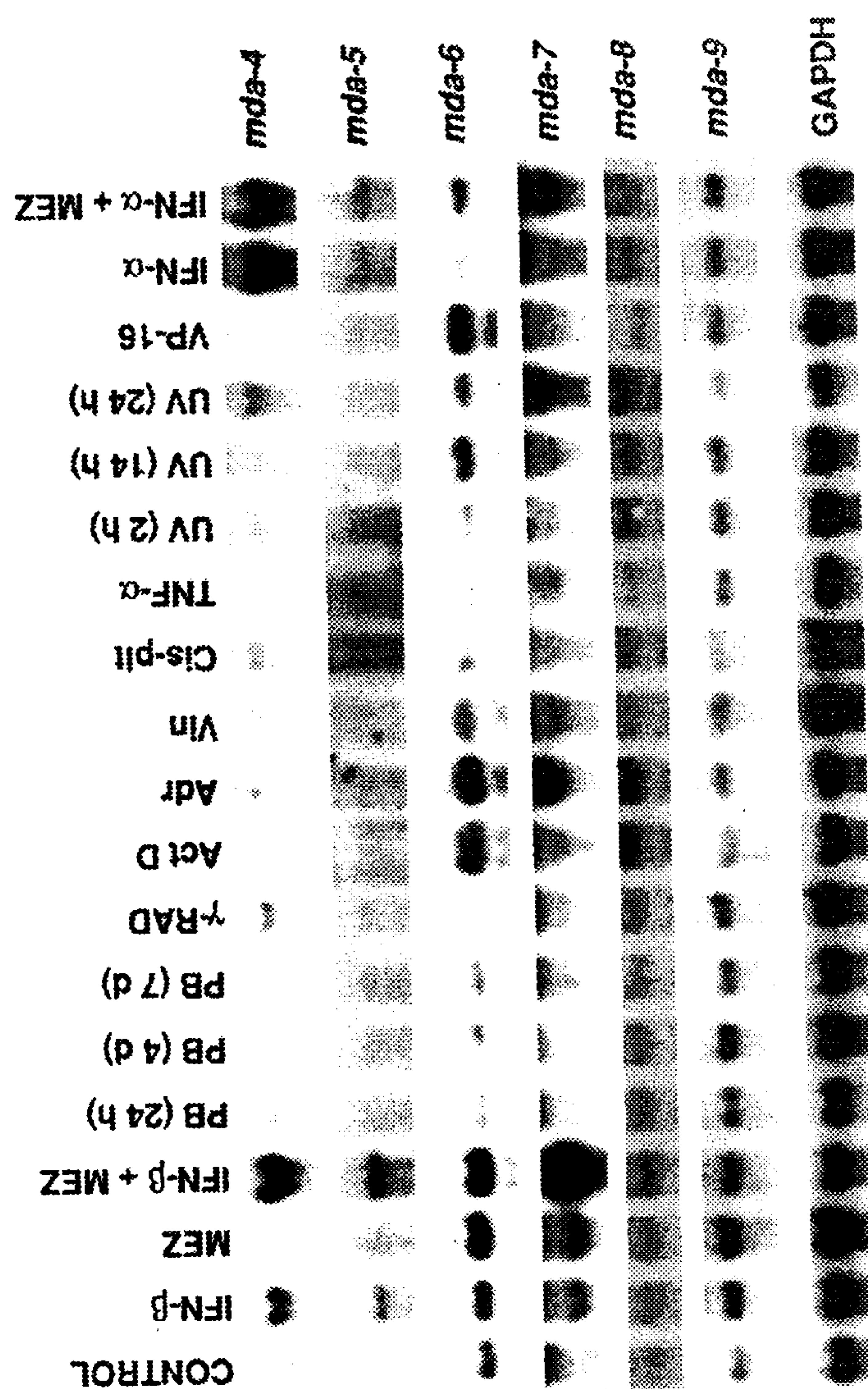
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FIGURE 18



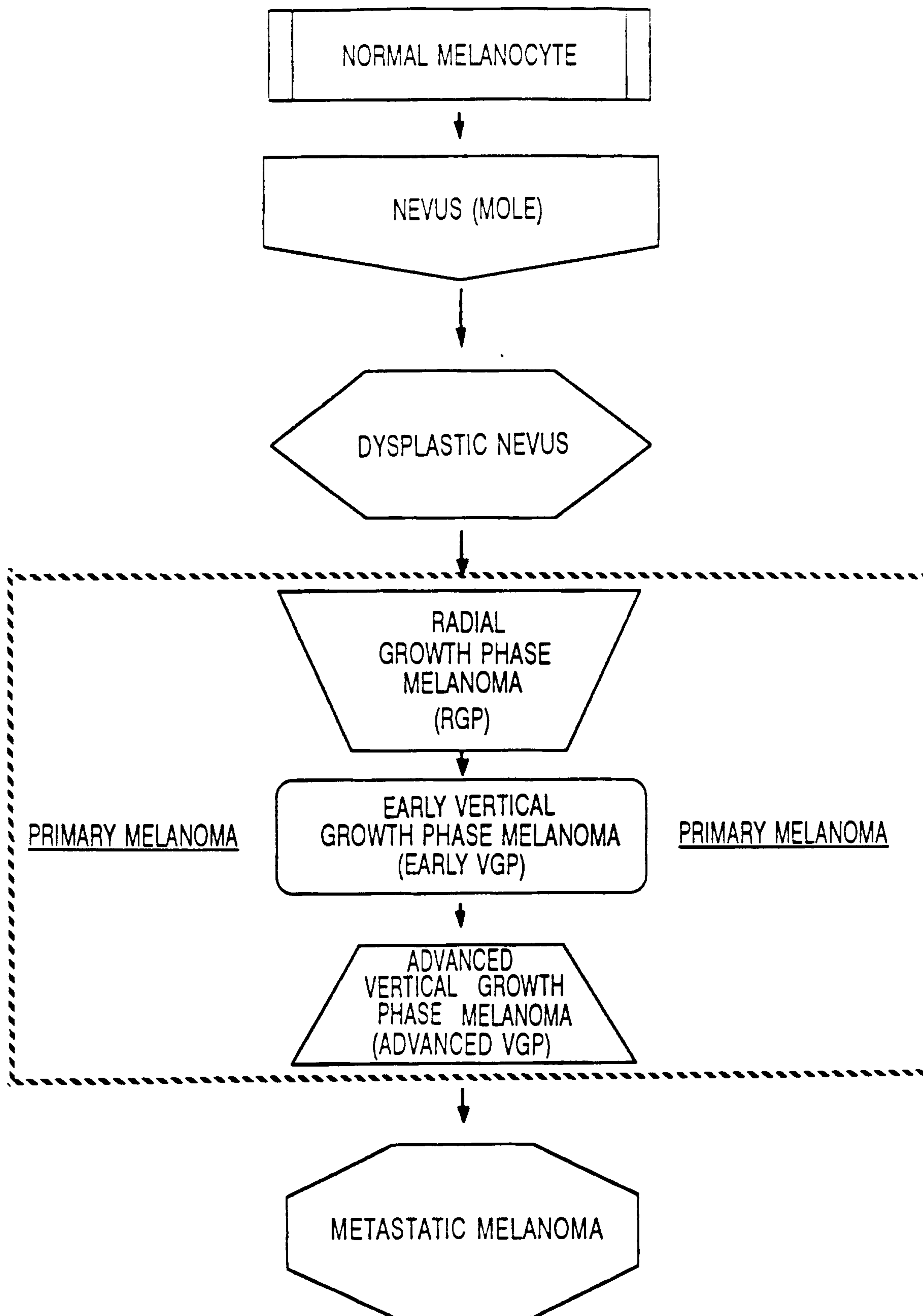
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FIGURE 19



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FIGURE 20



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FIGURE 21A FIGURE 21B FIGURE 21C FIGURE 21D

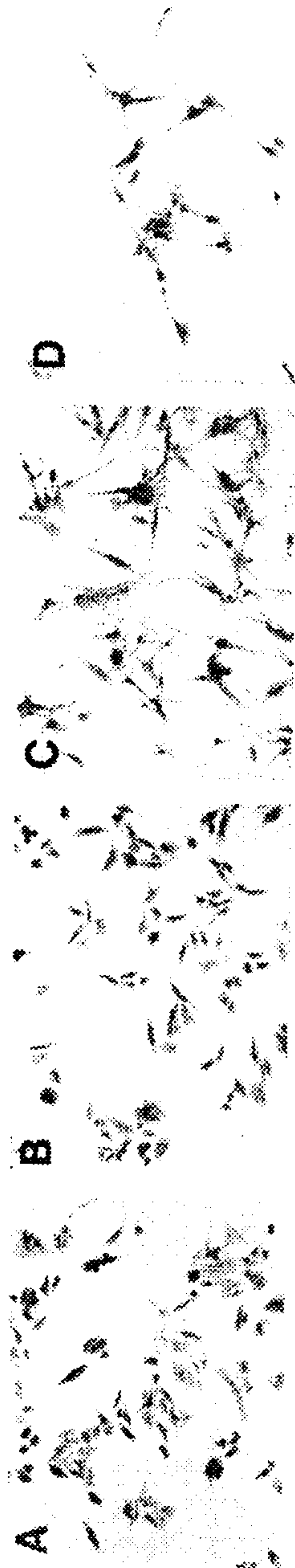


FIGURE 21E FIGURE 21F FIGURE 21G FIGURE 21H



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FIGURE 22

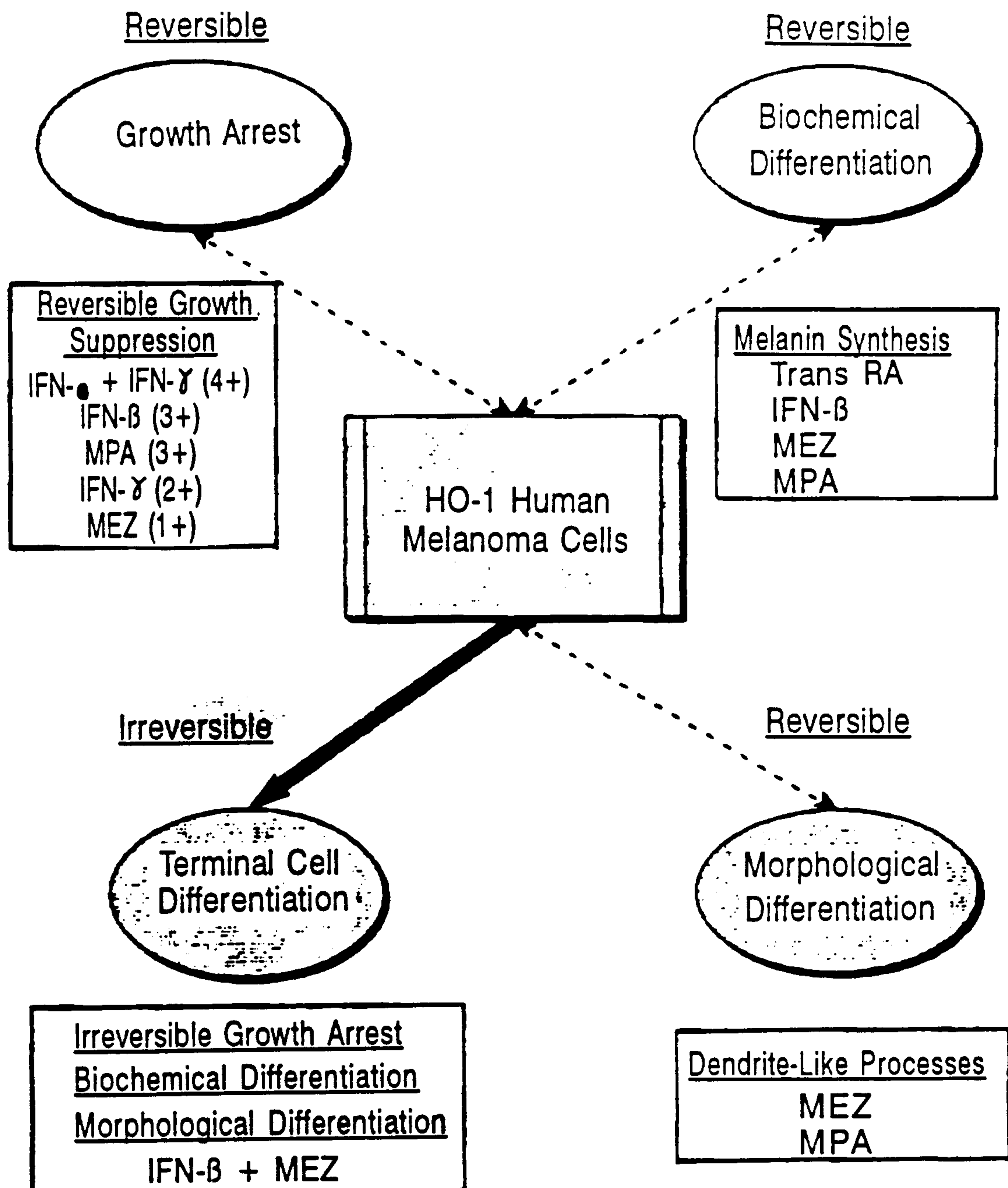


FIGURE 23A

FIGURE 23A
FIGURE 23B

AGCTGAGGTGTGAGCAGCTGCCGAAGTCAGTTCCTTGTGGAGCCGGAGCTGGGCGCGGAT 60
TCGCCGAGGCACCGAGGCACTCAGAGGAGCGCCCATGTCAGAACCGGCTGGGGATGTCCG 120
M S E P A G D V R
TCAGAACCCATGCGGCAGCAAGGCCCTGCCGCCCTCTTCGGCCCCAGTGGACAGCGAGCA 180
Q N P C G S K A C R L F G P V D S E Q
GCTGAGCCGCGACTGTGATGCGCTAATGGCGGCTGATCCAGGAGGCCCGTGAGCGATG 240
L S R D C D A L M A G C I Q E A R E R W
GAACTTCGACTTGTCAACCGAGACACCACTGGAGGCTGACTTCGCCCTGGGAGCGGTGCGG 300
N F D F V T E T P L E G D F A W E R V R
GGGCCTTGGCCTGCCCAAGCTCTACCTTCCCCACGGGGCCCCGGCAGGCCGGATGAGTT 360
G L G L P K L Y L P T G P R R G R D E L
GGGAGGAGGCAGCGGCCCTGGCACCTCACCTGCTCTGCTGCAGGGGACAGCAGGAAGA 420
G G G R R P G T S P A L L Q G T A E E D
CCATGTGGACCTGTCTACTGTCTGTACCTTGTGCCCTCGCTCAGGGGAGCAGGCTGAAGG 480
H V D L S L S C T L V P R S G E Q A E G
GTCCCCAGGTGGACCTGGAGACTCTCAGGGTCCGAAACGGCGCAGACCAGCATGACAGA 540
S P G G P G D S Q G R K R Q T S M T D
TTTCTACCACTCCAACGCCGGCTGATCTTCTCCAAGAGGAGCCCTAATCCGCCACAG 600
F Y H S K R R L I F S K R K P *
GAAGCCTGCAGTCCCTGGAGCGGAGGCCCTCAAGGCCCGCTCTACATCTTCTGCCTTA 660
GTCTCAGTTGTGTCTTAATTATTATTGTGTTTAAATTAACACCTCCTCATGTAC 720
ATACCCCTGGCCGCCCTGCCCCCAGCCTCTGGCATTAGAAATTATTAAACAATAACTA 780

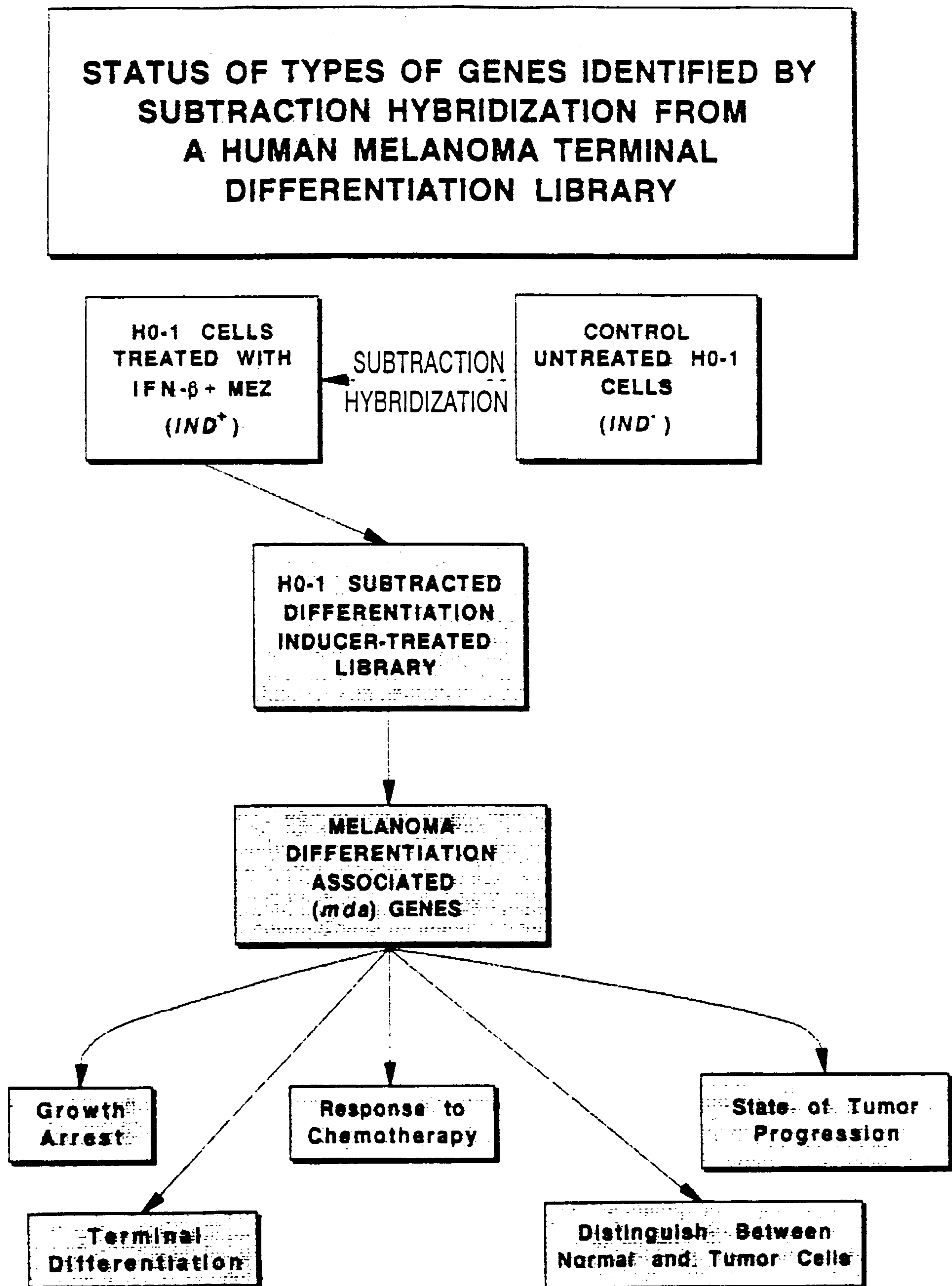
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FIGURE 23B

GGCGGTTGAATGAGAGGTTCCCTAAGAGTGCTGGGCATTTTATTTATGAAATACTATTT 840
AAAGCCTCCTCATCCCGTGTCTCCTTCTCCCGAGGTTGGTGGGCCGGCTT 900
CATGCCAGCTACTTCTCCTCCCACTTGTCCGCTGGGTACCCCTCTGGAGGGGTGTG 960
GCTCCTTCCCATCGCTGTCACAGGCGTTATGAATTCACCCCTTTCCTGGACACTCAG 1020
ACCTGAATTCTTTTCATTGAGAAGTAACAGATGGCACTTTGAAGGGCCTCACCGAG 1080
TGGGGGCATCATCAAAACTTTGGAGTCCCTCACCTCCTAAGGTTGGCAGGTGAC 1140
CCTGAAGTGAGCACAGCCTAGGGCTGAGCTGGGACCTGGTACCTCCTGGCTCTTGATA 1200
CCCCCCTCTGTGTGAAGCAGGGGAAGGTGGGTCCTGGAGCAGACCCCGCCT 1260
GCCCTCATGGCCCCCTCTGACCTGCACTGGGAGCCCCGTCTCAGTGTGAGCCTTTTCCCT 1320
CTTTGGCTCCCCCTGTACCTTTTGAGGAGCCCCAGCTACCCCTTCTCAGCTGGGCTCT 1380
GCAATTCCCCCTCTGCTGCTGCCCTTGTCCCTTCCCTTCAGTACCTCTCTCAGCT 1440
CCAGGTGGCTCTGAGGTGCCCTGTCCCAACCCCACTACCTCAGCTCAATGGACTGGAAGGGA 1500
AGGGACACACAAGAAGAAGGCACCCTAGTTCTACCTCAGGCAGCTCAAGCAGCGACCGC 1560
CCCCCTCCTAGCTGTGGGGTGAGGGTCCCATGTGGTGGCACAGGCCCTTGAGTGGG 1620
GTTATCTCTGTGTAGGGGTATATGATGGGGAGTAGATCTTCTAGGAGGGAGACACTG 1680
GCCCCCTCAAATCGTCCAGCGACCTTCCCTCATCCCACTCCCTCCCACTTCATTGCAC 1740
TTTGATTAGCAGCGGAACAAGGAGTCAGACATTTAAGATGGTGGCAGTAGAGGCTATGG 1800
ACAGGGCATGCCACGTGGGCTCATATGGGGCTGGGAGTAGTGTCTTCTGGCACTAAC 1860
GTTGAGCCCCCTGGAGGCACTGAAGTCTTAGTGTACTTGGAGTATTGGGCTCTGACCCCA 1920
AACACCTTCCAGCTCCTGTAACTAGTGGCCTGGACTGTTTCTCTCGGCTCCCCATGTG 1980
TCCTGGTTCCTGTTCTCCACCTAGACTGTAAACCTCTCGAGGGCAGGACACACCCCTG 2040
TACTGTTCTGTGCTCTTTCACAGCTCCTCCACAAATGCTGAATAAACAGCGTCTCAAT 2100
AAATGATTCTTAGTGACTTTAAAAAAAATAAAAAAAA 2147

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FIGURE 24



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FIGURE 25A

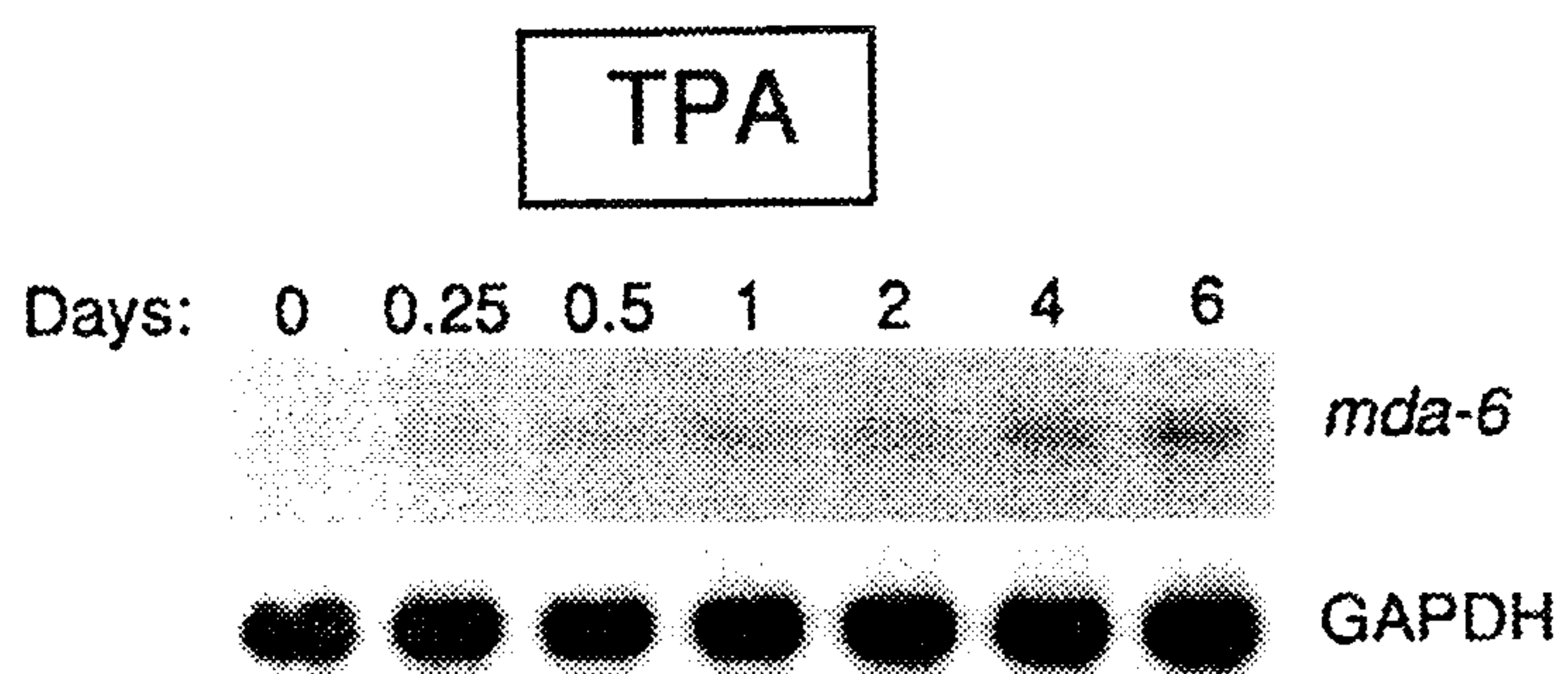
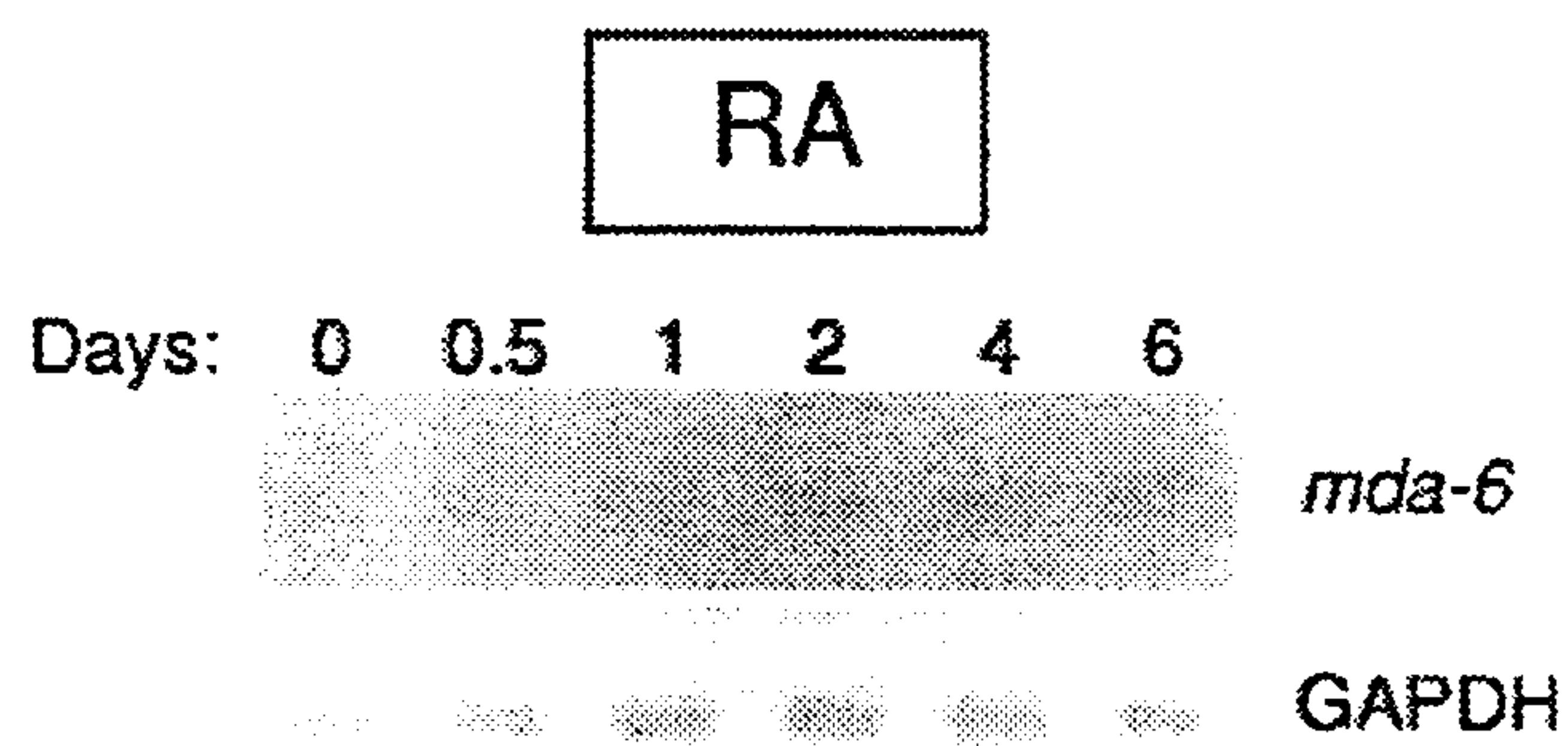


FIGURE 25B



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FIGURE 26A

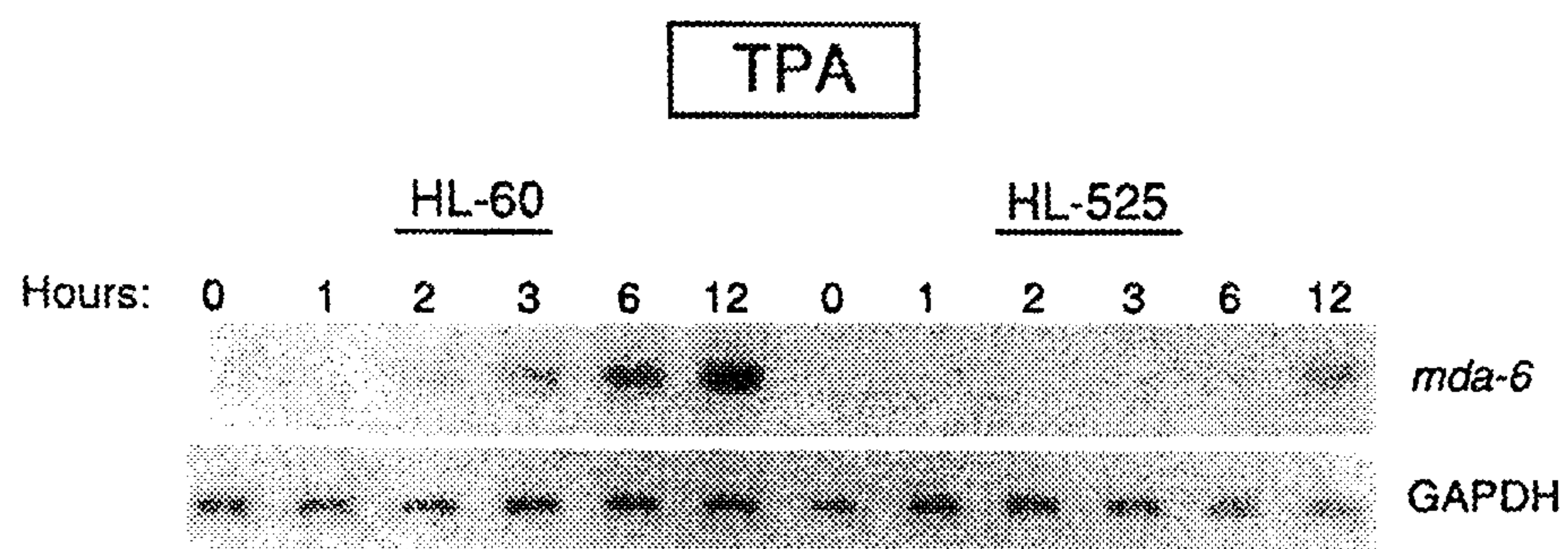


FIGURE 26B

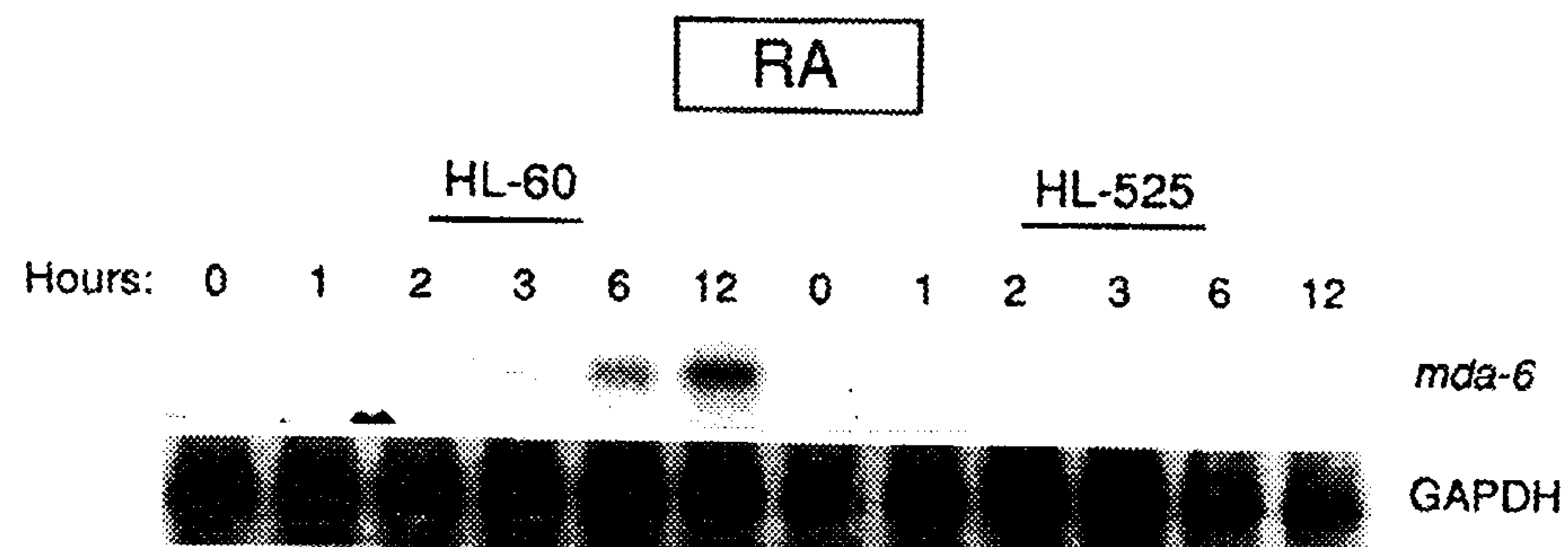
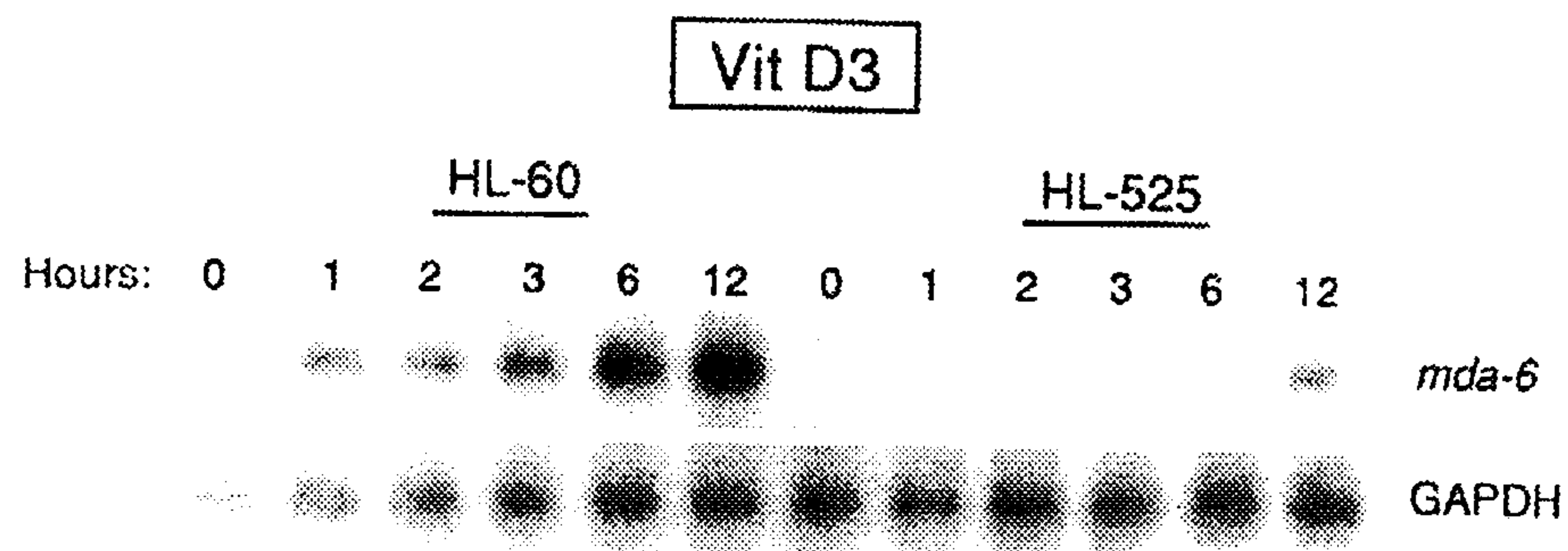


FIGURE 26C



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FIGURE 27A

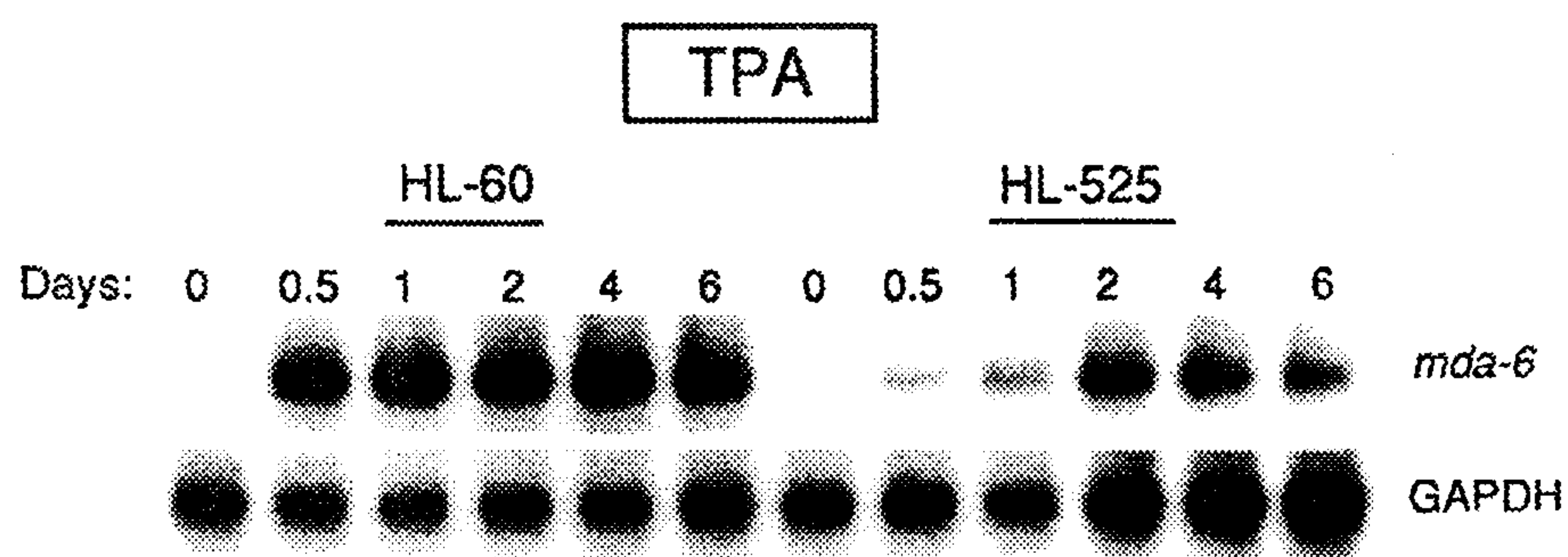


FIGURE 27B

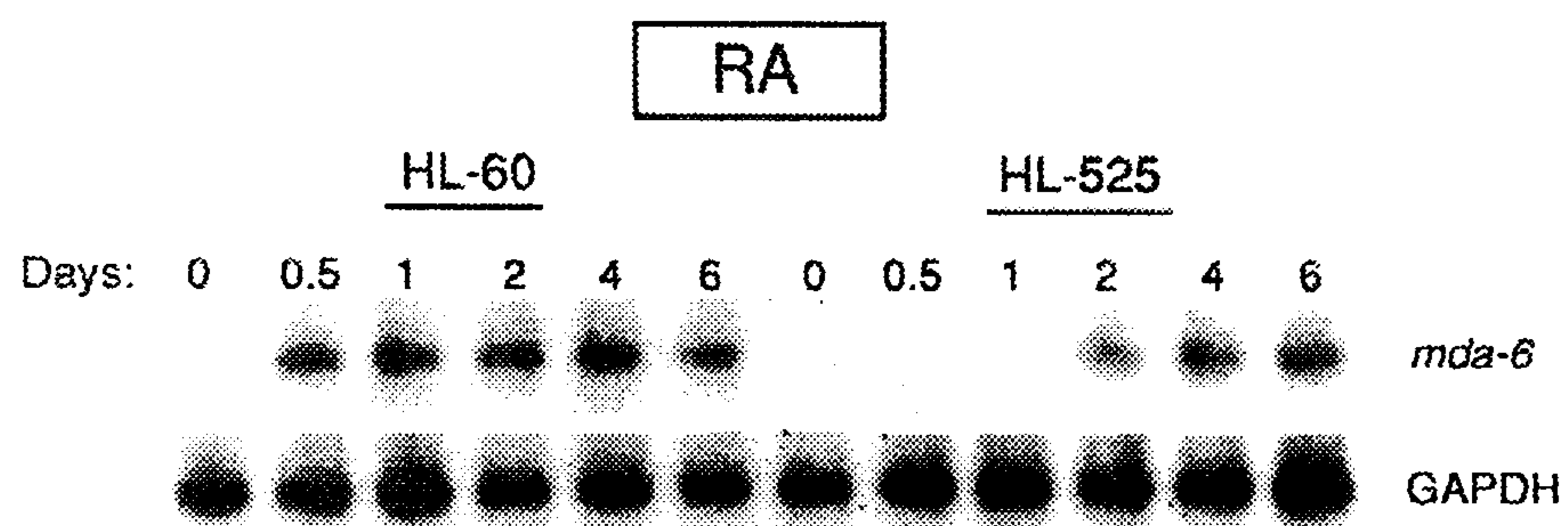
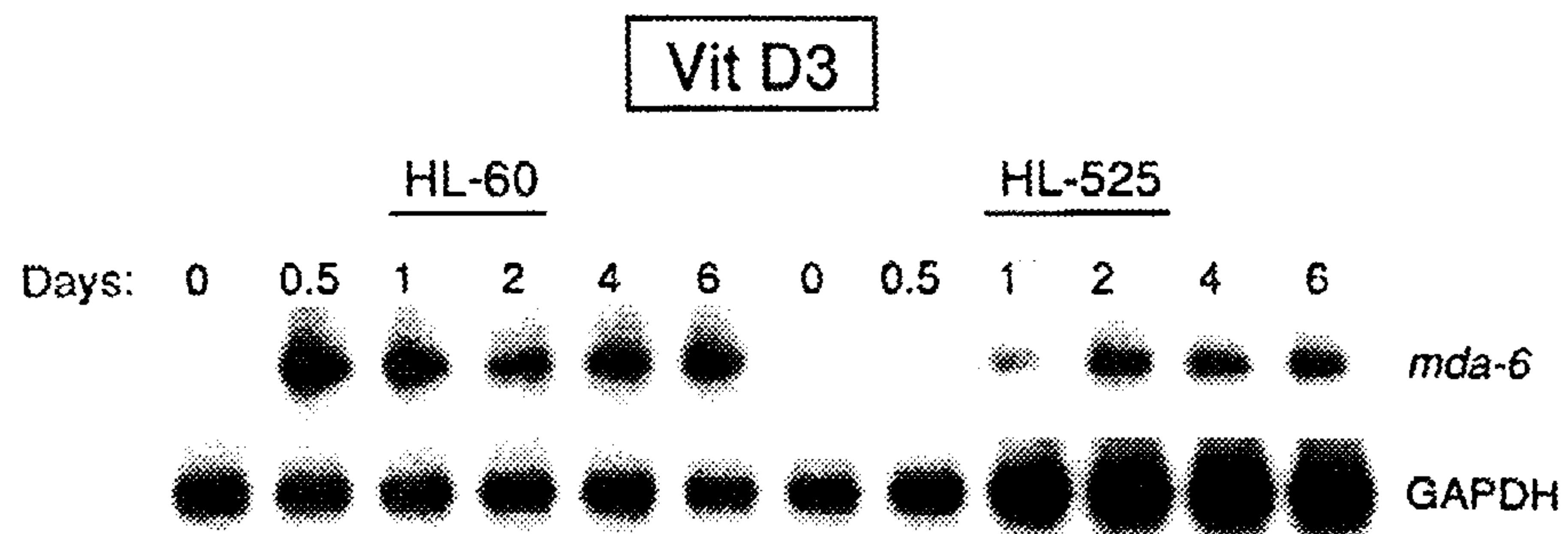
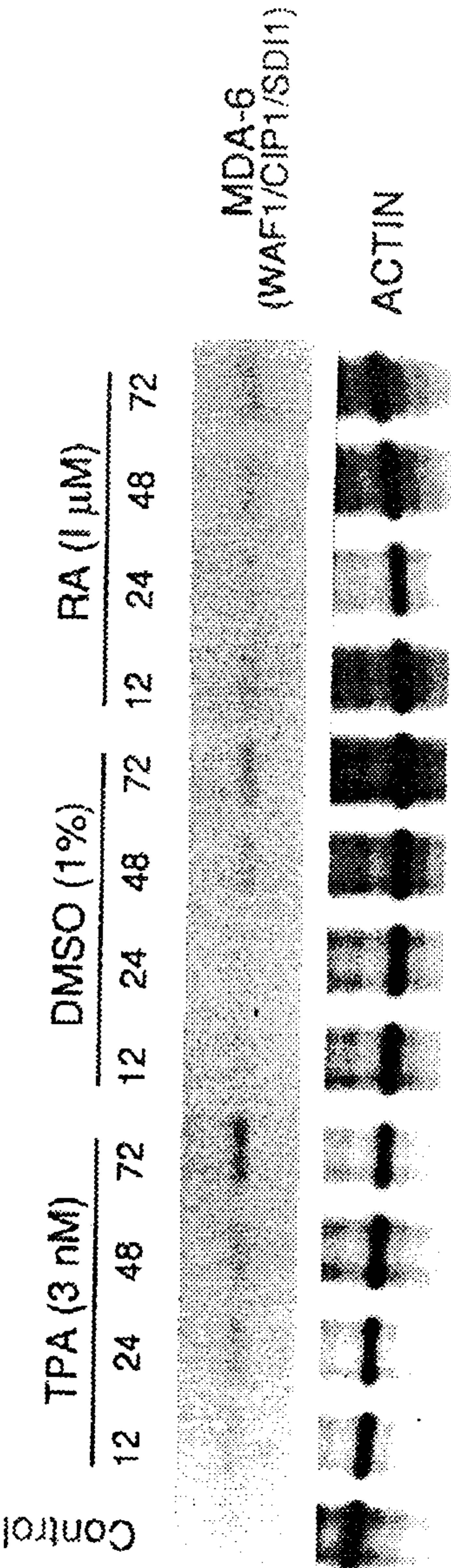


FIGURE 27C



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FIGURE 28



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FIGURE 29A

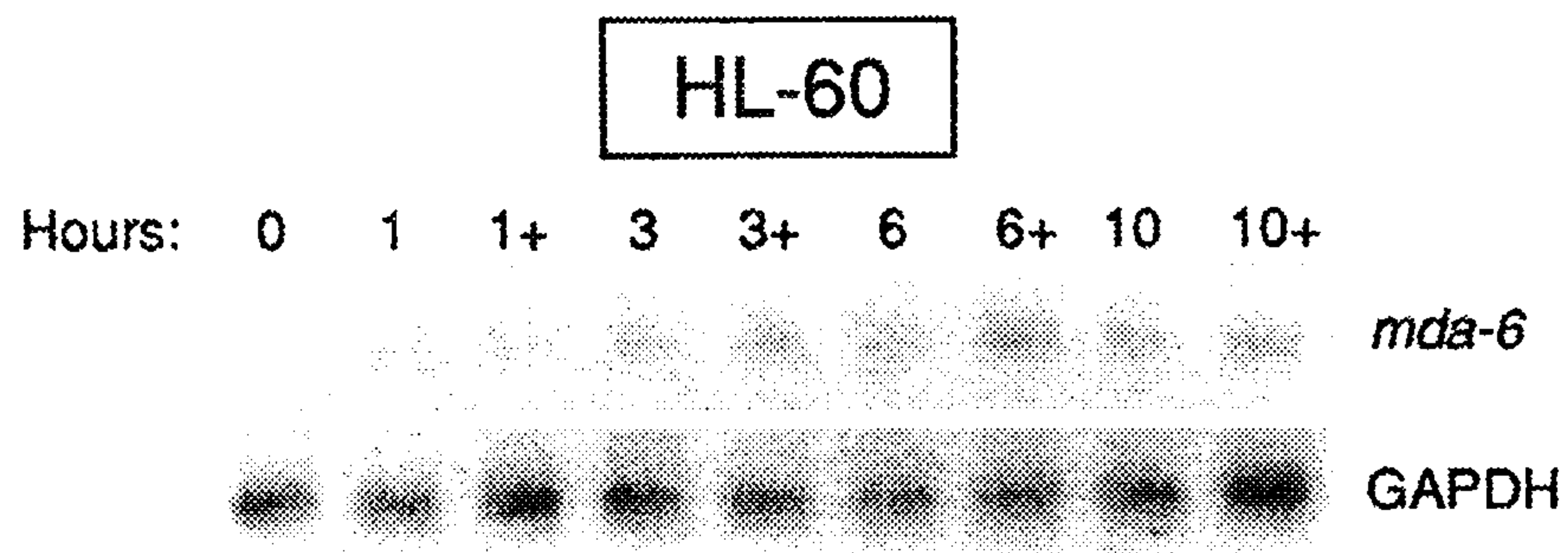
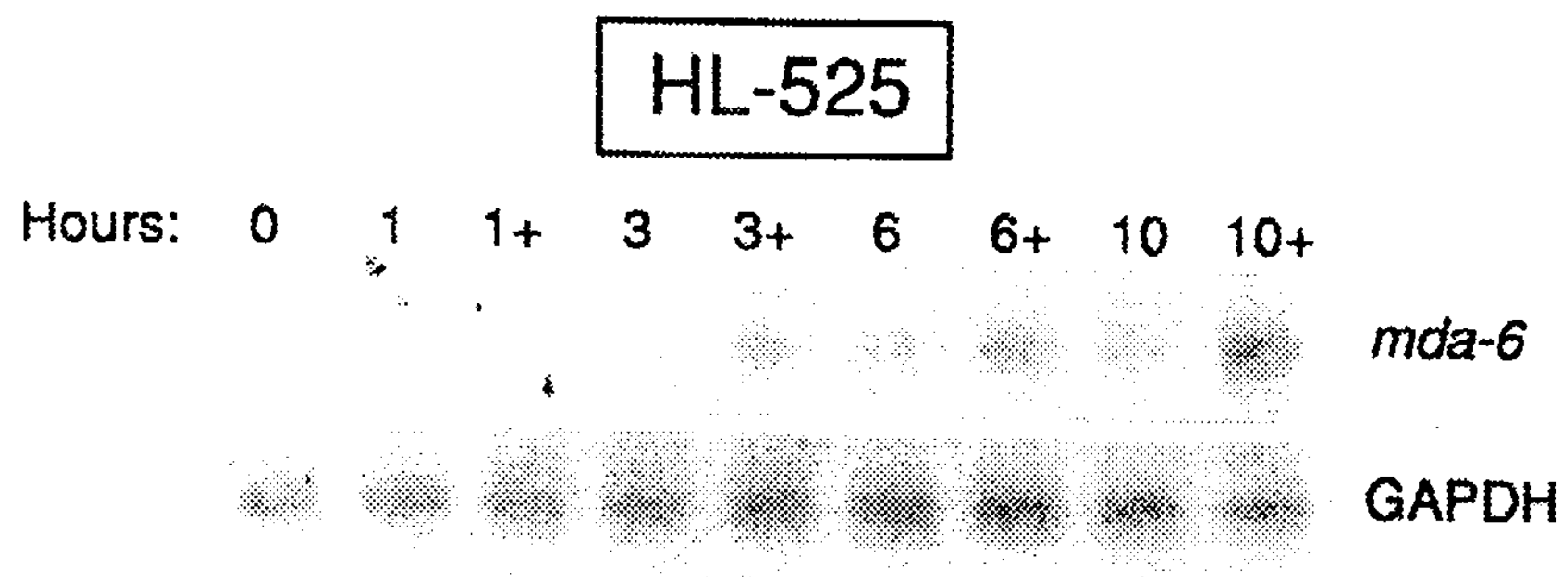


FIGURE 29B



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FIGURE 30

ATGTCAGAACCGGCTGGGATGTCCGTCAAGAACCCATGCGGCAGCAAGGCCCTGCCCGCCG
M S E P A G D V R Q N P C G S K A C R R
CTCTTCGGCCAGTGGACAGCAGCTGAGCCGCGACTGTGATGCGCTAATGGCGGCG
L F G P V D S E Q L S R D C D A L M A G
TGCAATCCAGGAGGCCCGTGAGCGATGGAACTTCGACTTTGTACCGAGACCACTGGAG
C I Q E A R E R W N F D F V T E T P L E
GGTGAATTCGCCCTGGAGCGTGTGCGGGCCCTTGGCCCTGCCCAAGCTCTACCTTCCCACG
G D F A W E R V R G L G L P K L Y L P T
GGGCCCGGCGAGGCCGGATGAGTTGGGAGGAGGCAGCGGCCCTGGCACCTCACCTGCT
G P R R G R D E L G G R R P G T S P A
CTGCTGCAGGGACAGCAGAGGAAGACCATGTGGACCTGTCACTGTCTTGTACCCCTTGTG
L L Q G T A E E D H V D L S L S C T L V
CCTCGCTCAGGGAGCAGGCTGAAGGTCCCAGGTGGACCTGGAGACTCTCAGGGTCGA
P R S G E Q A E G S P G G P G D S Q G R
AAACGGCGCAGACCAGCATGACAGATTCTACCACTCCAACGCCGGCTGATCTTCTCC
K R R Q T S M T D F Y H S K R R L I F S
AAGAGGAAGCCCTAA
K R K P *

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FIGURE 31A

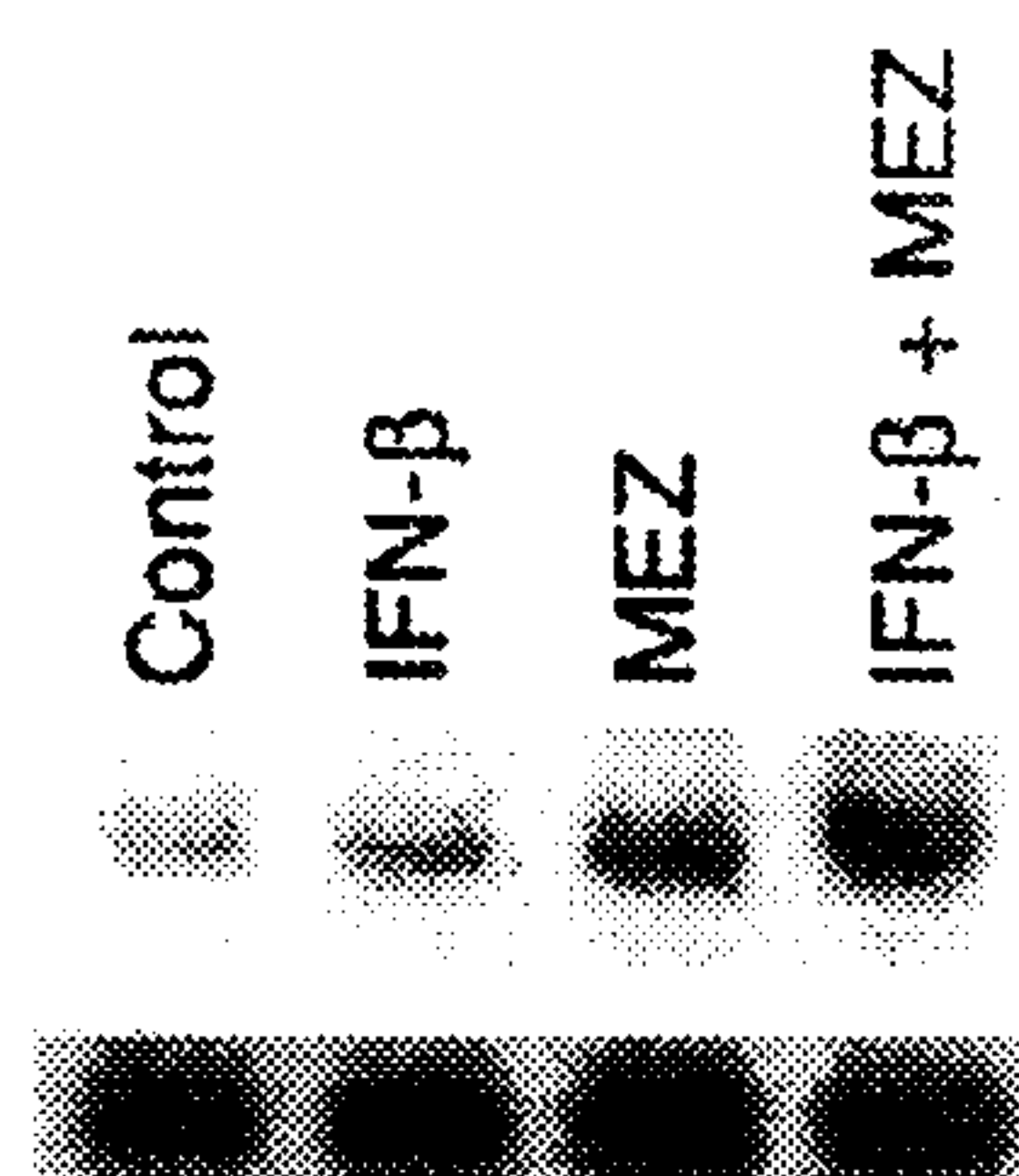


FIGURE 31B

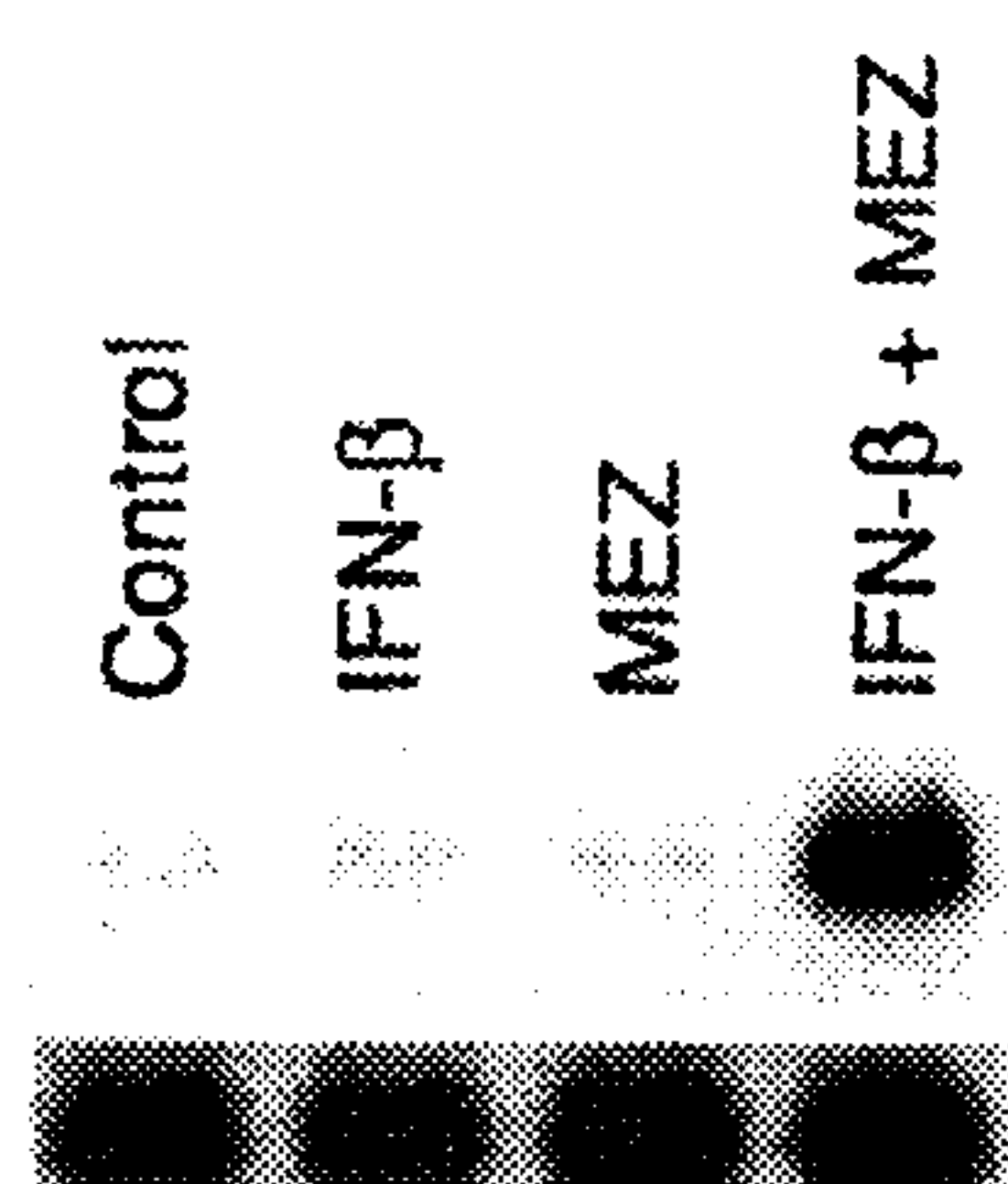


FIGURE 31C

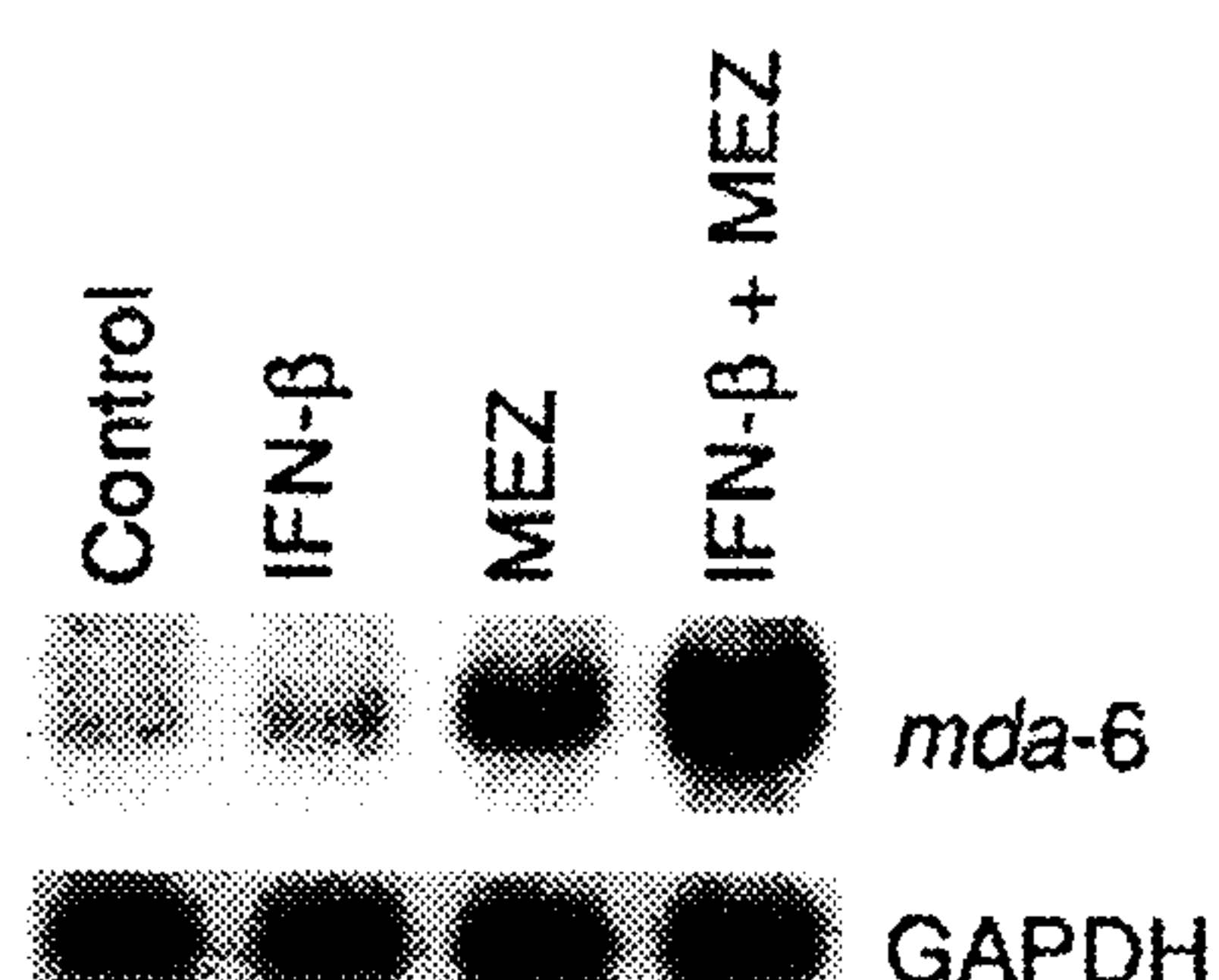


FIGURE 31D

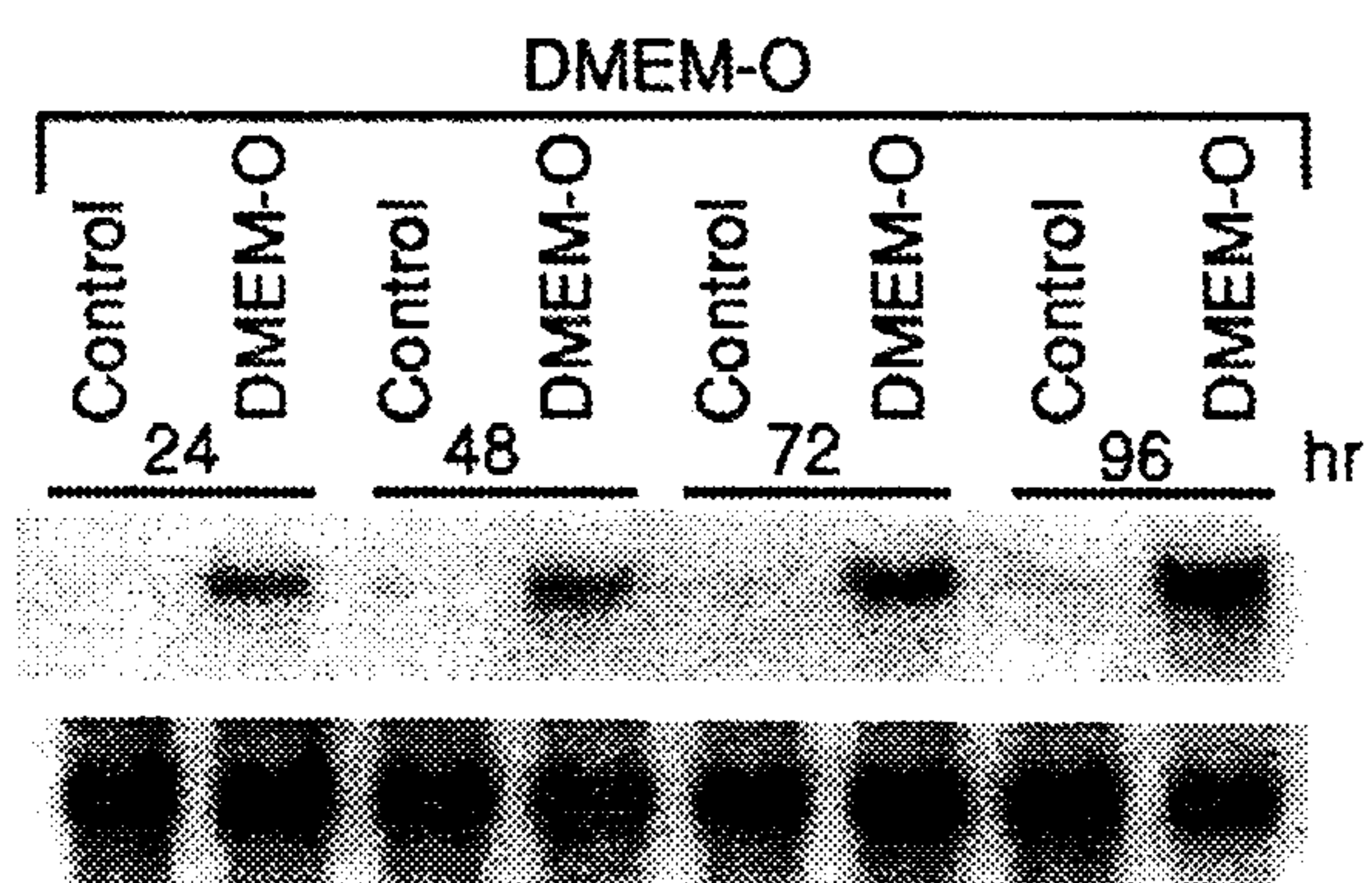
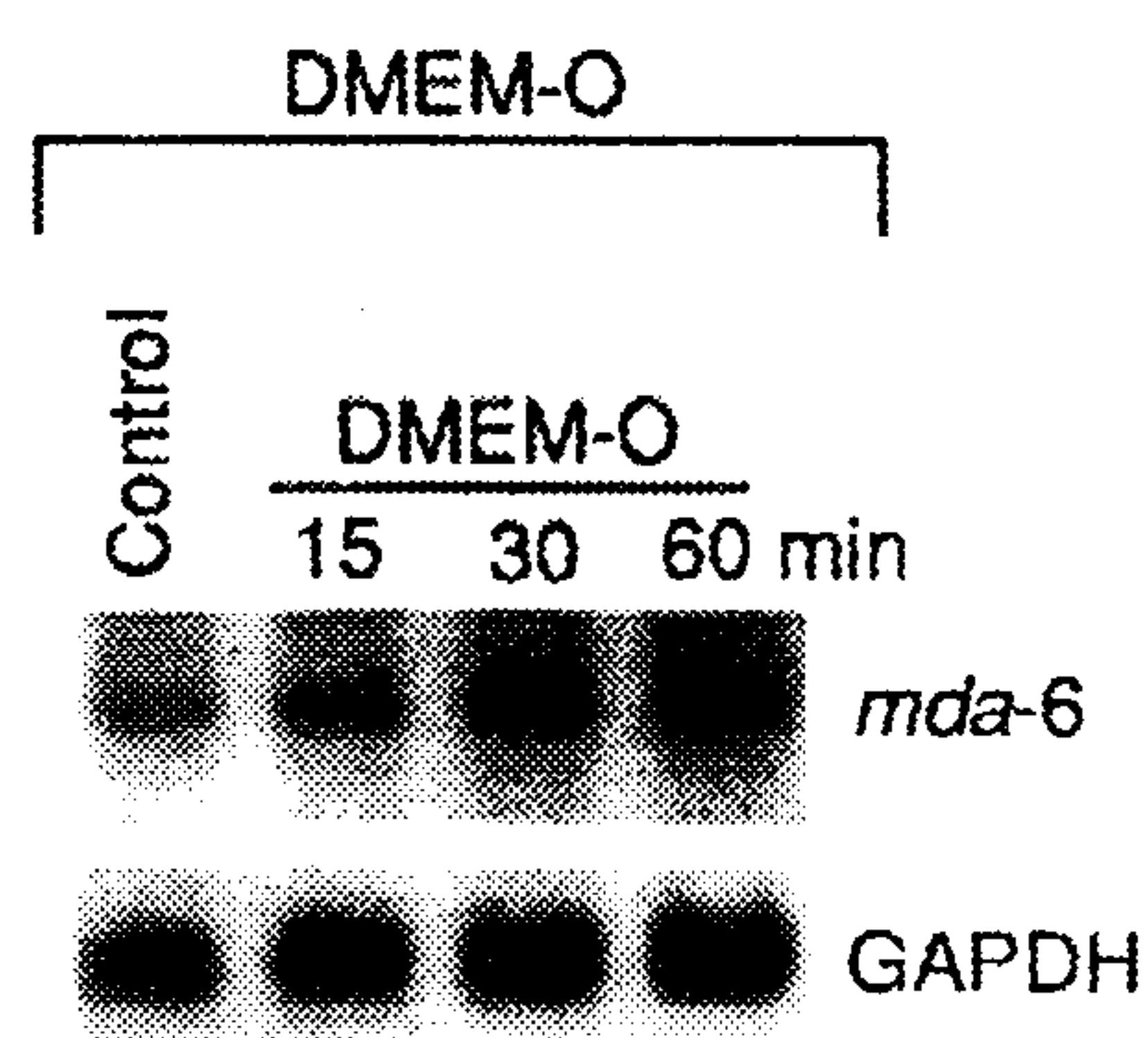
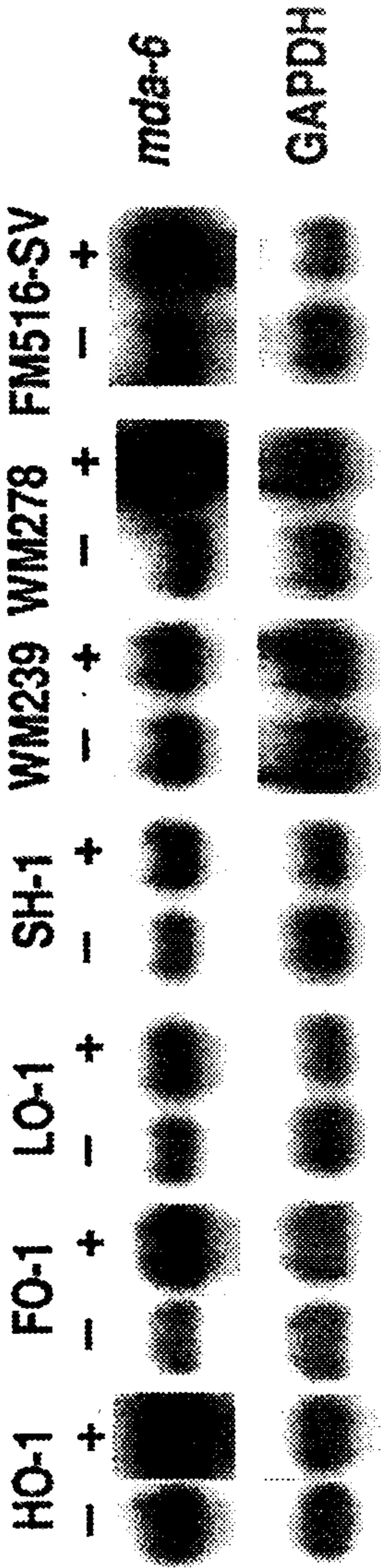


FIGURE 31E



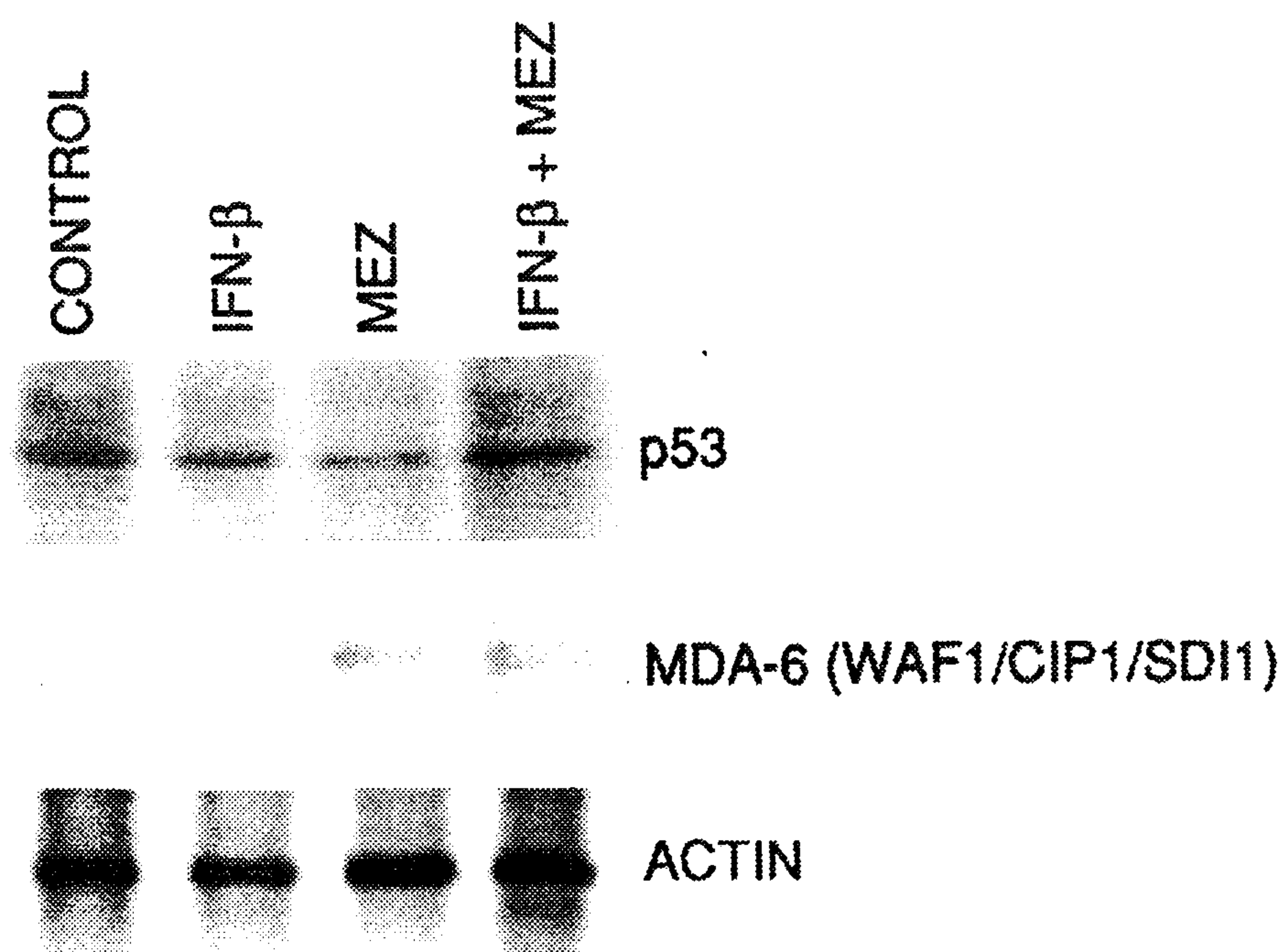
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FIGURE 32



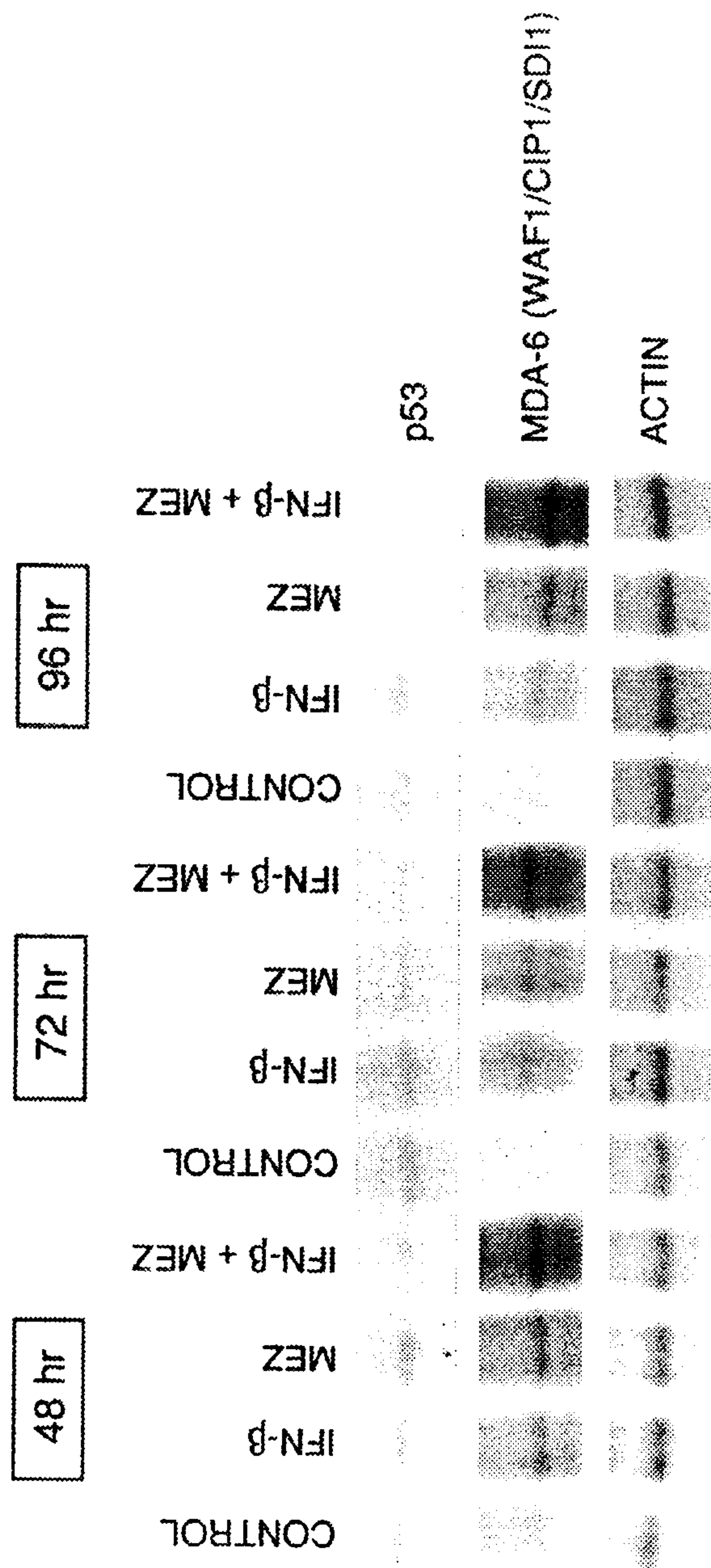
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FIGURE 33



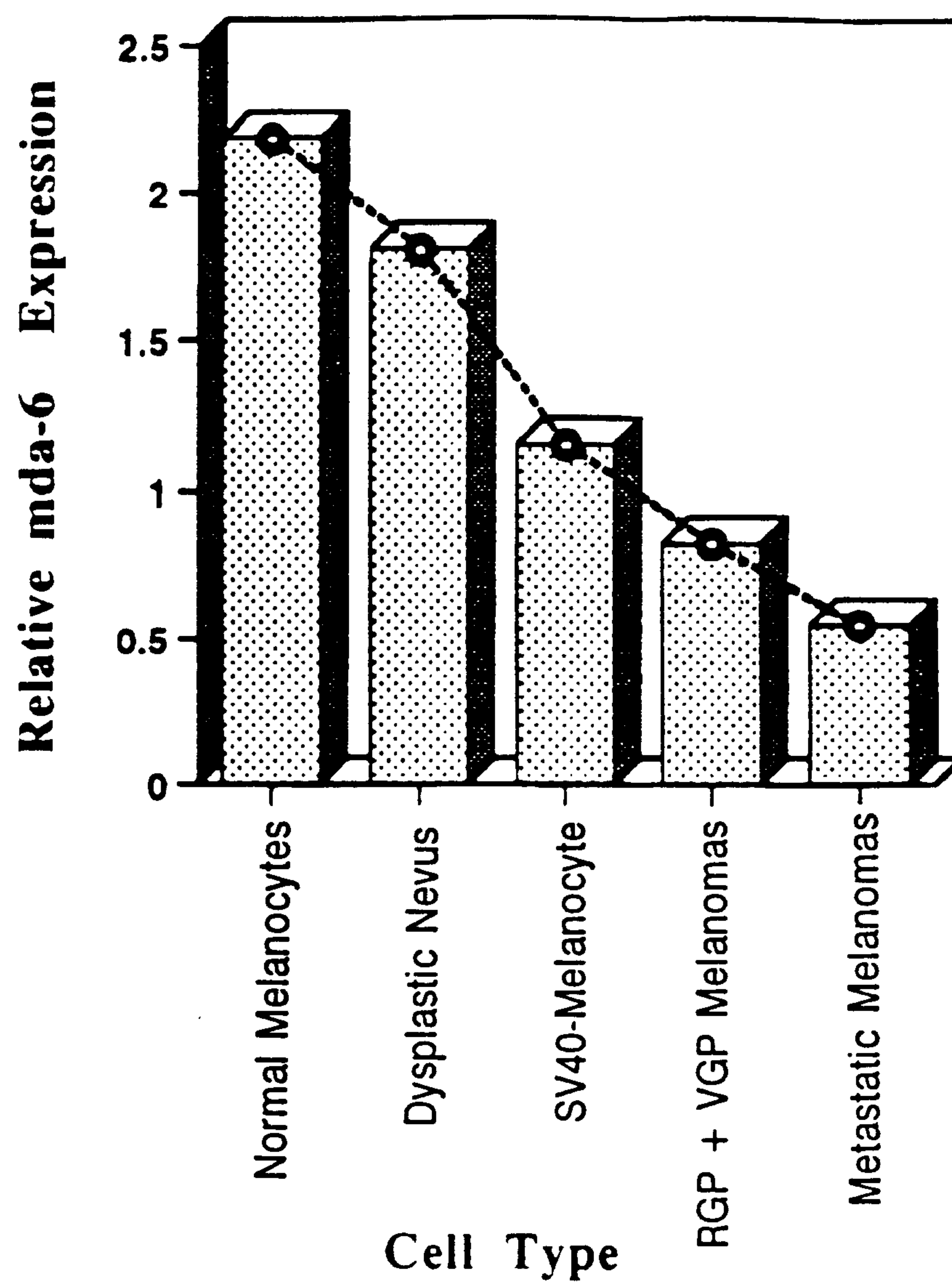
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FIGURE 34



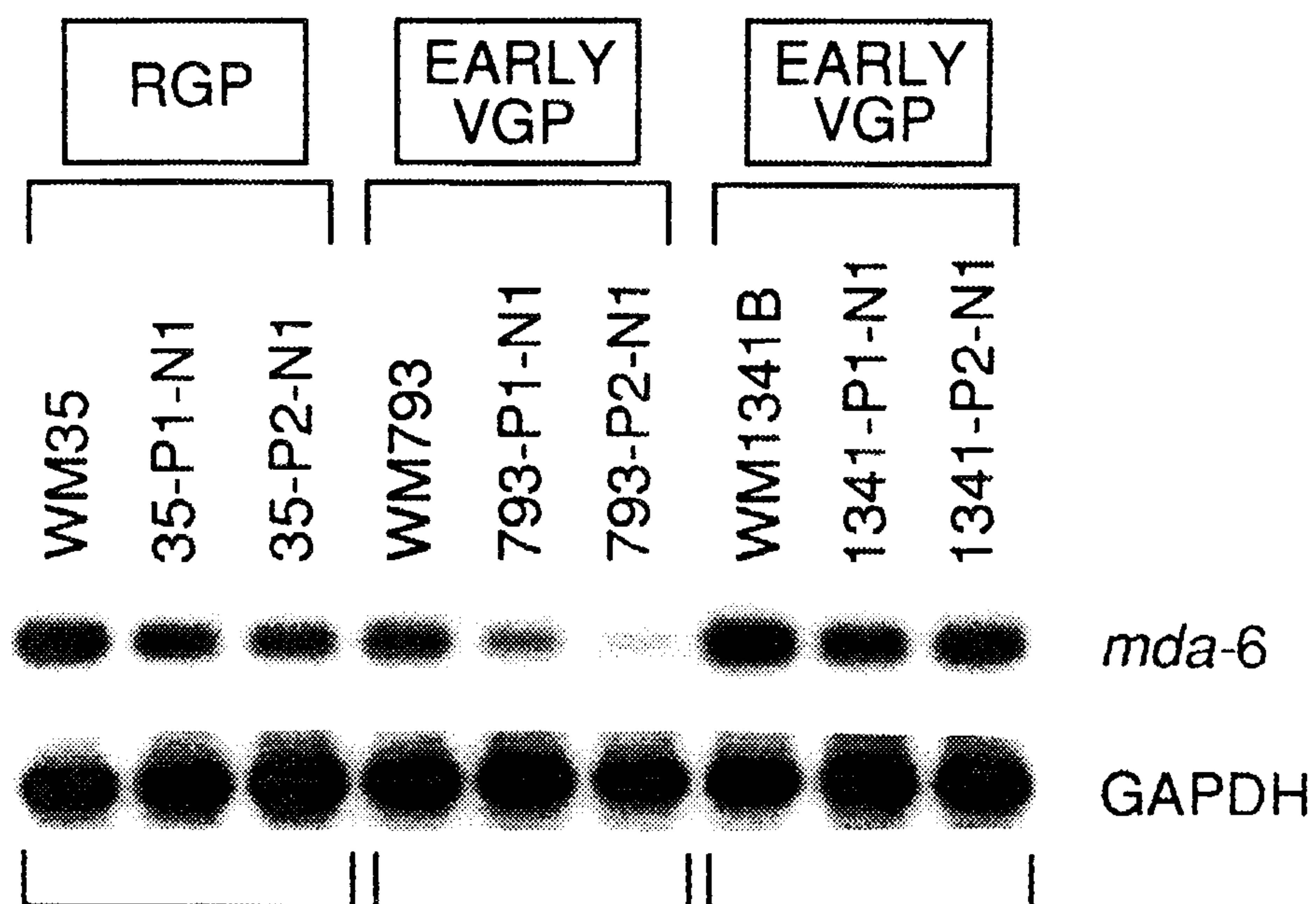
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FIGURE 35



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FIGURE 36



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FIGURE 37

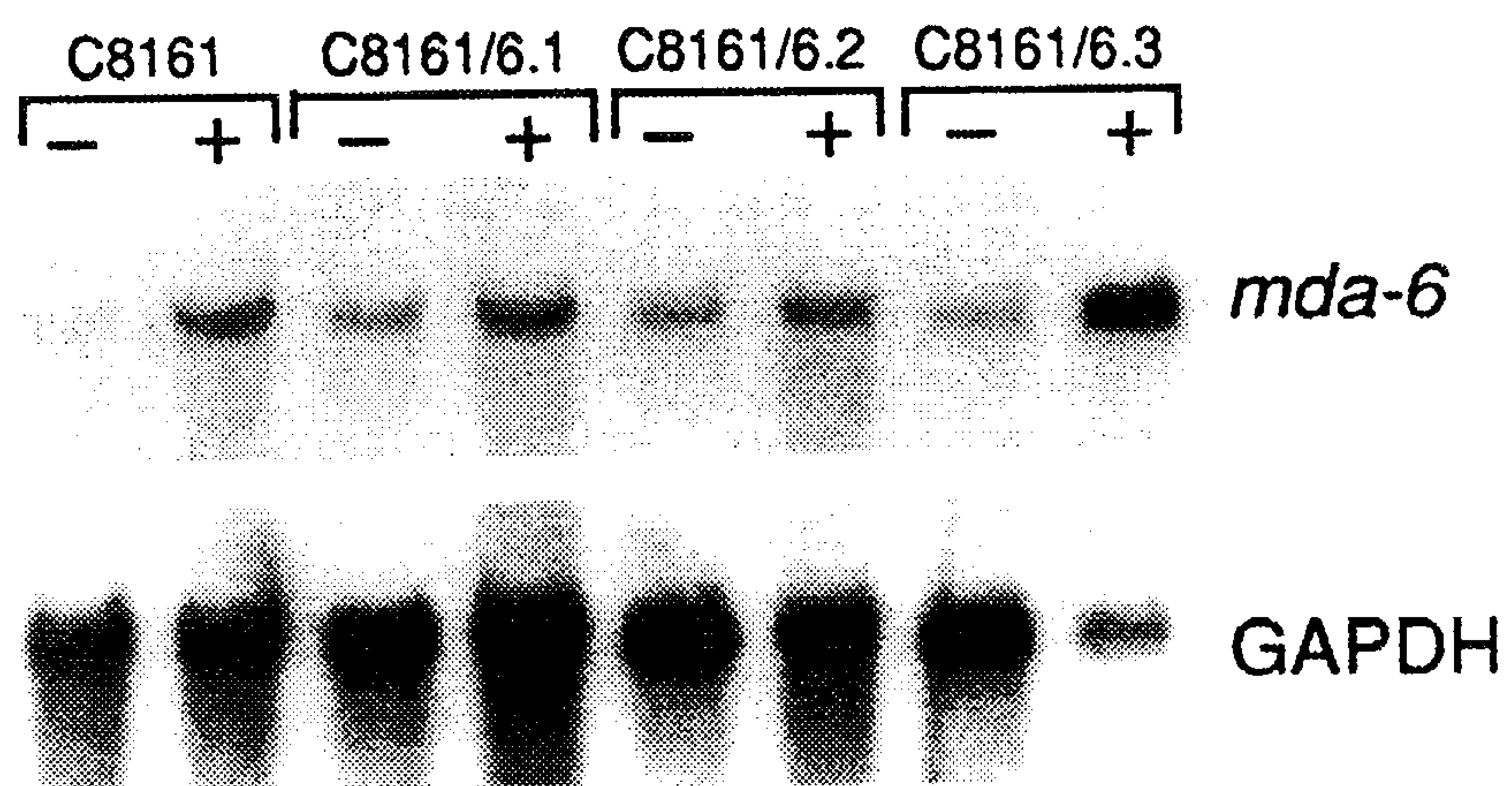


FIGURE 38A

FIGURE 38A
FIGURE 38B

MDA-7 . Seq
CTTGCCCTGCAACCTTTACTTCTGAAATGACTTCCACGGCTGGGACGGGAACCTTCCACC 60
CACAGCTATGCCCTCTGATTGGTGAATGGTGAAGTGCCCTGTCTAACTTTTCTGTAAAAAG 120
AACCAGCTGCCCTCCAGGCAGCCCTCAAGCATCATTACAGGACCAGAGGGACAAGA 180
CATGACTGTGATGAGGAGCTGCTTTCGCCAATTTAACACCAAGAAGAAATTGAGGCTGCTT 240
GGGAGGAAGGCCAGGAGGAACACGAGACTGAGAGATGAATTTCACAGAGGCTGCAAAG 300
M N F Q Q R L Q S

CCTGTGGACTTTAGCCAGACCCCTTCTGCCCTCCTTTTGCTGGCGACAGCCCTCTCAAATGCA 360
L W T L A R P F C P P L L A T A S Q M Q

GATGGTTGTGCTCCCTTGCCCTGGGTTTACCCCTGCTTCTGGAGCCAGGTATCAGGGGC 420
M V V L P C L G F T L L L W S Q V S G A

CCAGGGCCAAGAAATCCACTTTGGGCCCTGCCAAGTGAAGGGGTTGTTCCTCCAGAAACT 480
Q G Q E F H F G P C Q V K G V V P Q K L

GTGGGAAGCCTTCTGGGCTGTGAAGACACTATGCAAGCTCAGGATAACATCACGAGTGC 540
W E A F W A V K D T M Q A Q D N I T S A

CCGGCTGCTGCAGCAGGAGGTTCTGCAGAACGTCTCGGATGCTGAGAGCTGTACCTTGT 600
R L L Q Q E V L Q N V S D A E S C Y L V

CCACACCCCTGCTGGAGTTCTACTTGAAAACCTGTTTCAAAAACCTACCACAATAGAACAGT 660
H T L L E F Y L K T V F K N Y H N R T V

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FIGURE 38B

TGAAGTCAGGACTCTGAAGTCA	TTCTACTCTGGCCAACTTTGTTCTCATCGTGTC	720
E V R T L K S F S T L A N N F V L I V S		
ACAACTGCAACCCAGTCAAGAAATGAGATGTTTCCATCAGAGACAGTGCACACAGGCG	780	
Q L Q P S Q E N E M F S I R D S A H R R		
GTTTCTGCTATTCCGGAGAGCATTCAAACAGTTGGACGTAGAAAGCAGCTCTGACCAAAGC	840	
F L L F R R A F K Q L D V E A A L T K A		
CCTTGGGAAGTGGACATTCTTGACCTGGATGCAGAAATTCTACAAGCTCTGAATGTC	900	
L G E V D I L L T W M Q K F Y K L *		
TAGACCAGGACCTCCCTCCCTGGCACTGGTTTGTTCCTGTGTCA	960	
CCCTTCCCTATGCTGTTCACCTGGACACTTCACGCCCTTGCCCATGGTCCCATTCTTGCC	1020	
CAGGATTATTGTCAAAGAAGTCATTCTTTAAGCAGGCCAGTGACAGTCAGGGAAGTGC	1080	
CTCTGGATGCTGTGAAGAGTCTACAGAGAAGATTCTTGATTTATTACAACCTCTATTTAA	1140	
TTAATGTCAGTATTTCAACTGAAGTCTATTATTGTGAGACTGTAAGTTACATGAAGG	1200	
CAGCAGAAATATTGTGCCCCCATGCTTTACCCCTCACAAATCCTTGCCACAGTGTTGGGC	1260	
AGTGGATGGTGCTTAGTAAGTACTTAATAAACTGTGTGCTTTTGTGGCCTGTCTTTG	1320	
GATTGTTAAAAACAGAGAGGGATGCTTGGAATGTAAAACTGAACCTTCAGAGCATGAAAT	1380	
CACACTGCTGTGATATCTGCAGGACAGAGCATTGCGGTGGGTAAAGTGATCTGT	1440	
TTGAAAAGTAACGATAAAATGTGGATTAAAGTGCCACACAAGCAGATCCTCAATAA	1500	
ACATTTCAATTTCCACCCACACTCGCCAGCTCACCCCATCCCTTTCCCTTGGTGCCC	1560	
TCCTTTTATCCCTAGTCATTCTCCCTAATCTTCCACTTGAGTGTCGAAGCTGACC	1620	
TTGCTGATGGTGACATTGCACCTGGATGTAATCCAAATCTGTGATGACATTCCCTGCTA	1680	
ATAAAAGACAACATAACTCAAAAAA	1718	

FIGURE 39

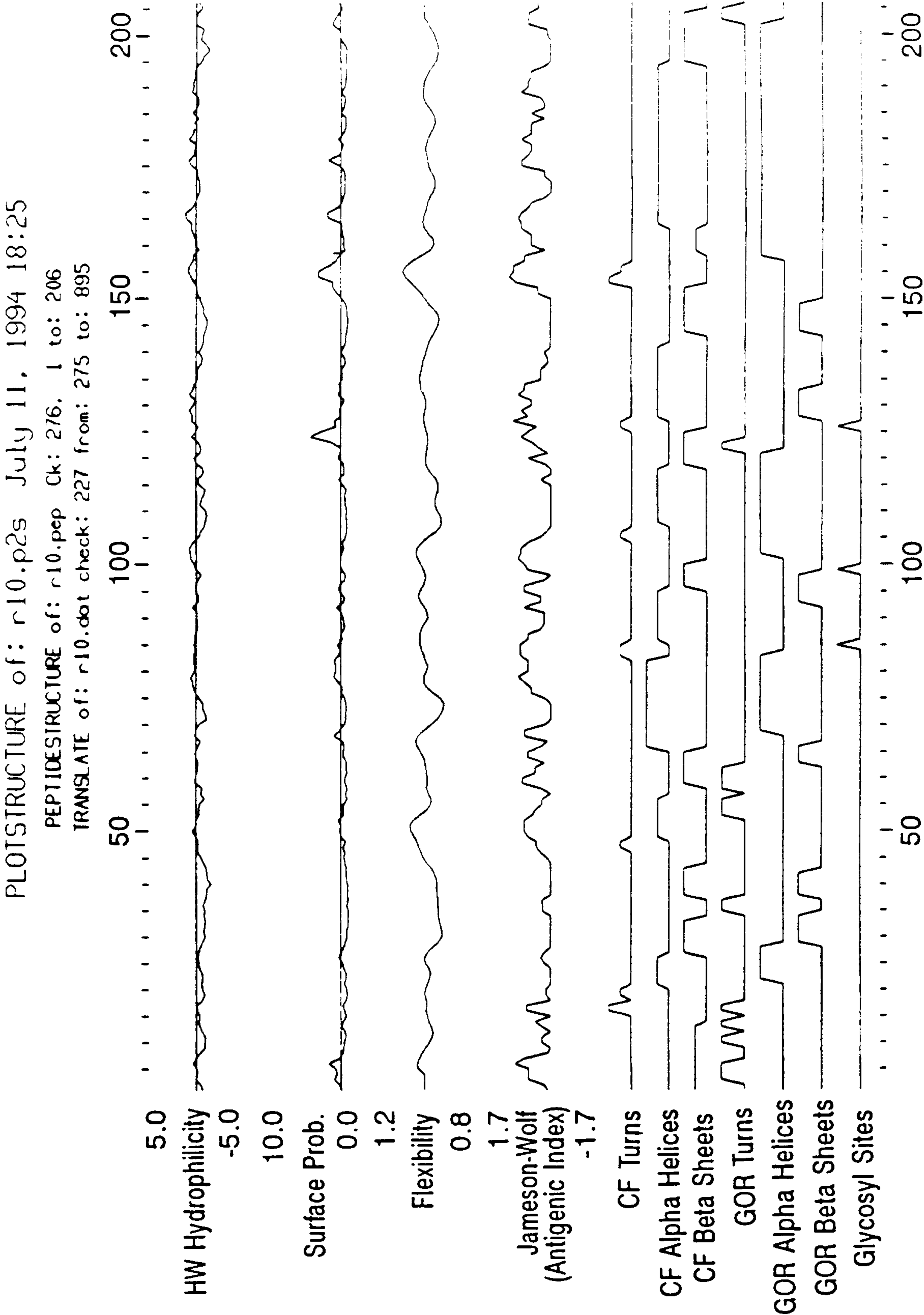
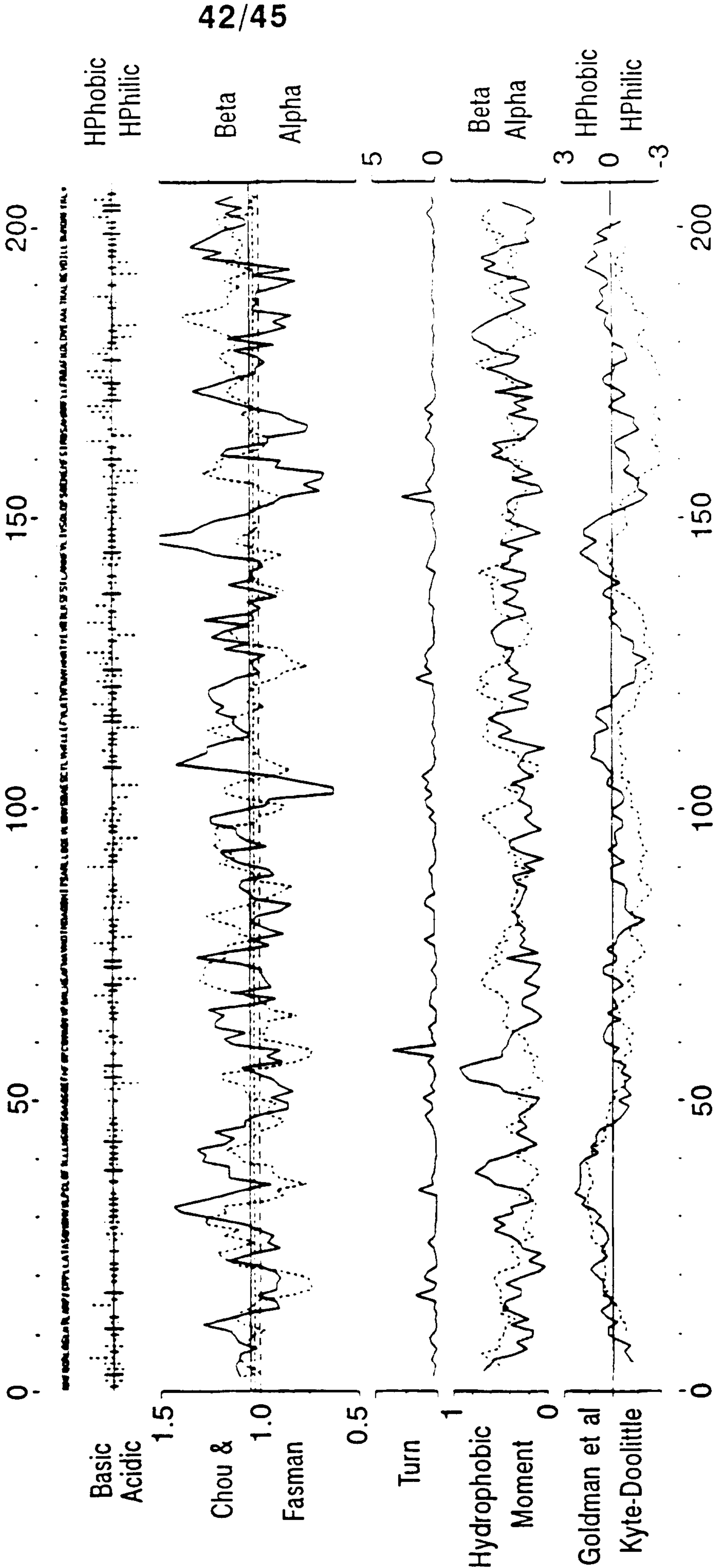


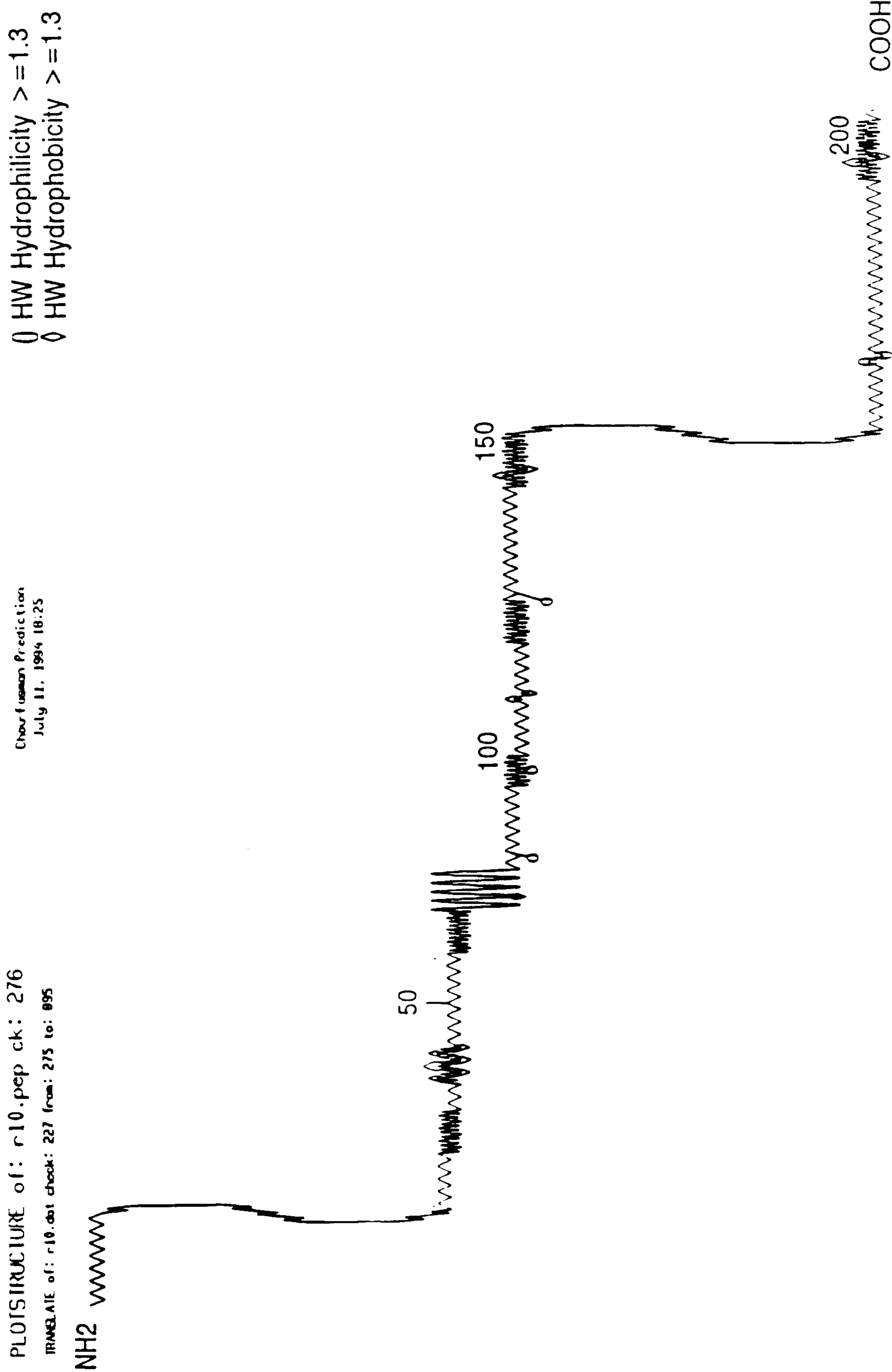
FIGURE 40

PEPPILOT of: R10.Pep ck: 276, 1 to 207 July 15, 1994 08:24
TRANSLATE of: r10.dat ck: 227 from: 275 to: 895



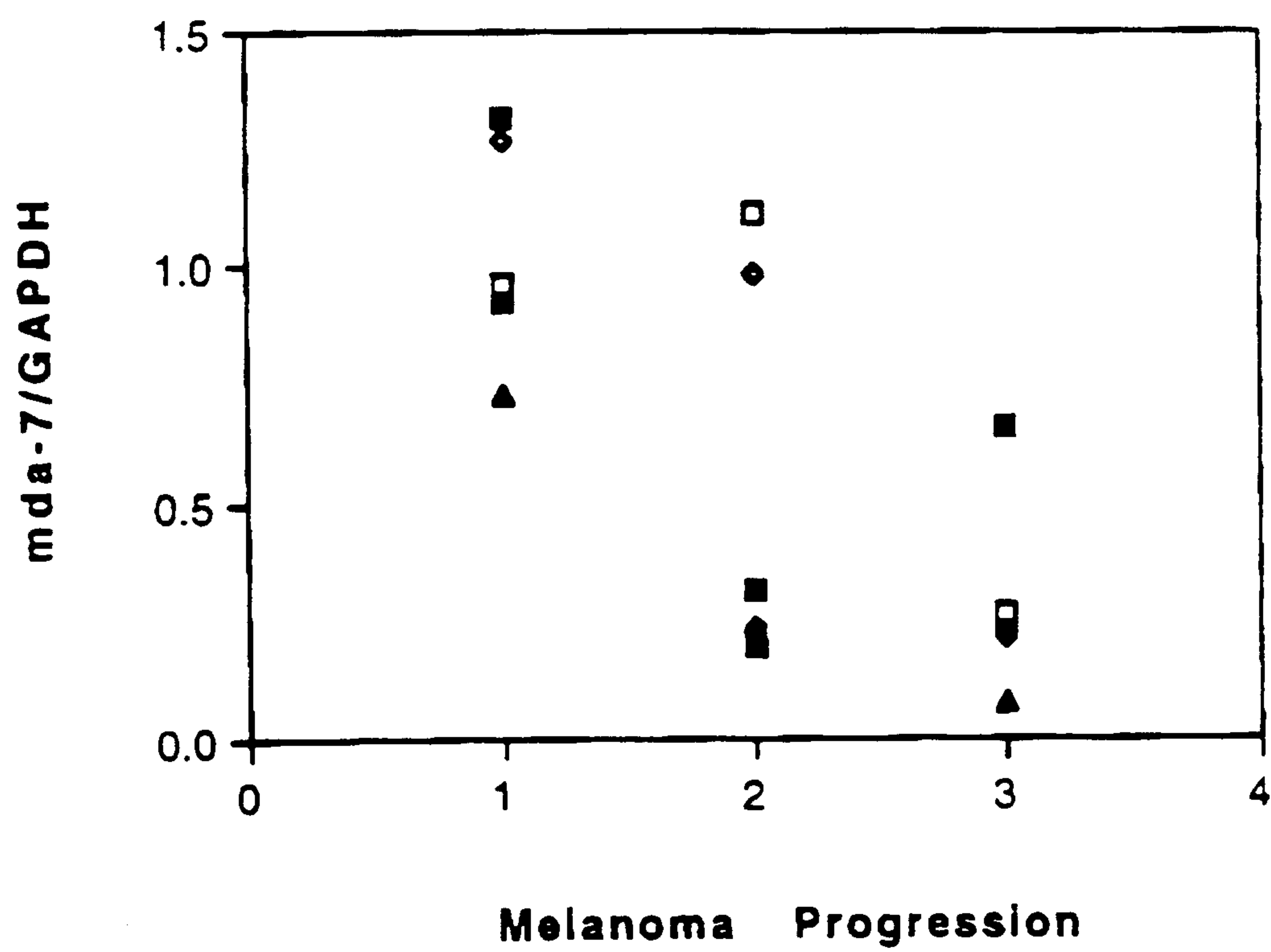
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FIGURE 41



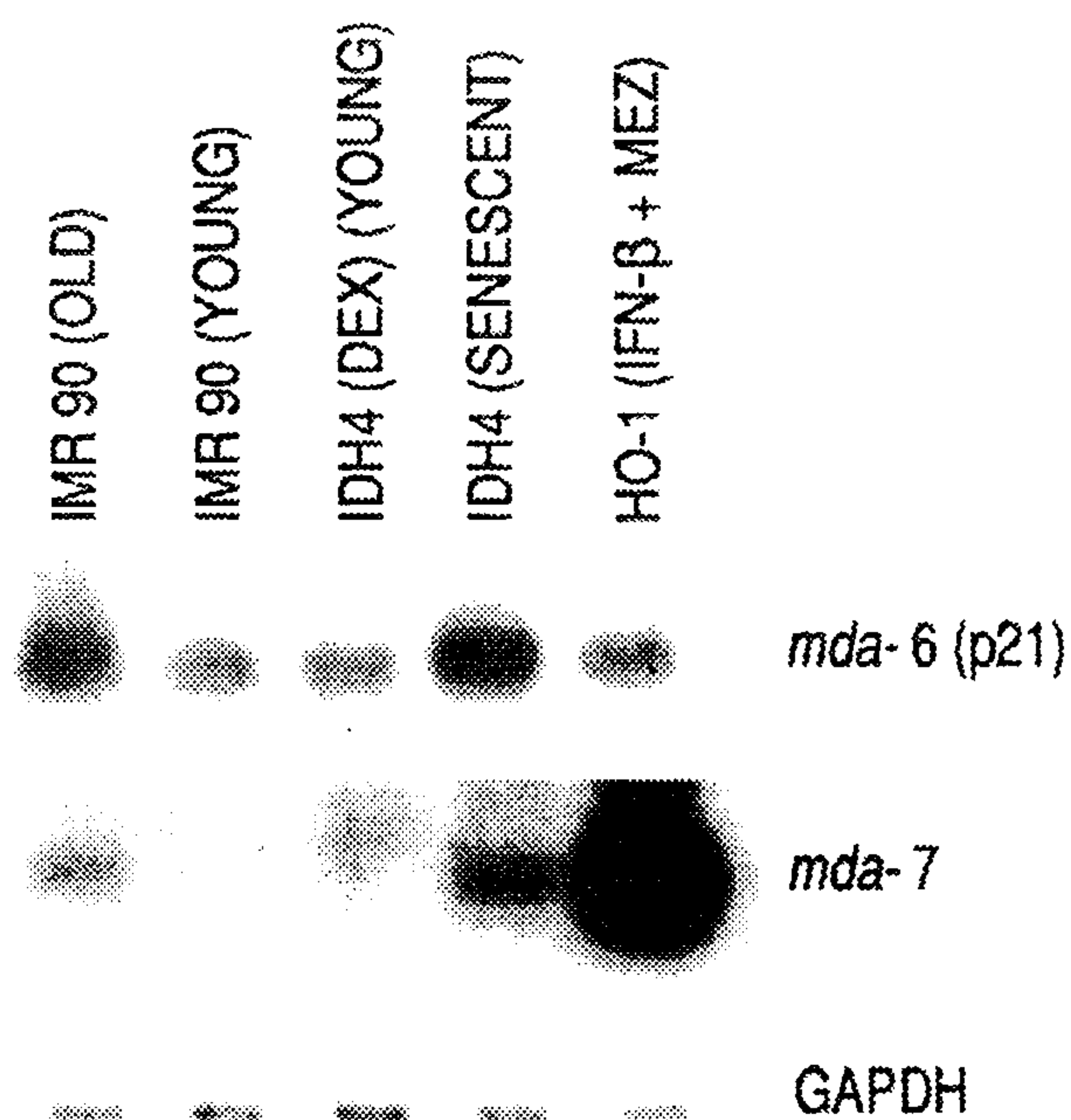
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FIGURE 42



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FIGURE 43



MELANOMA

NORMAL
CEREBELLUM

GLIOBLASTOMA
MULTIFORME

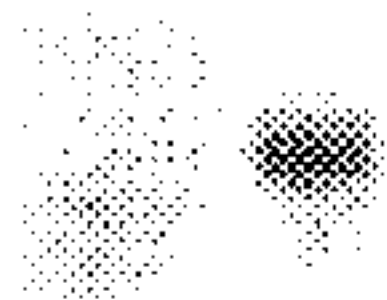
NORMAL SKIN
FIBROBLASTS

- +

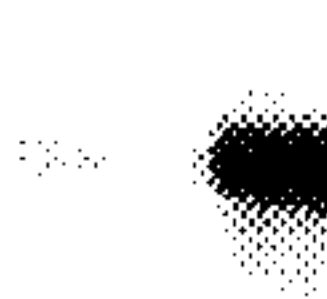
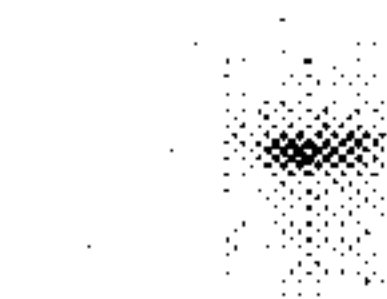
- +

- +

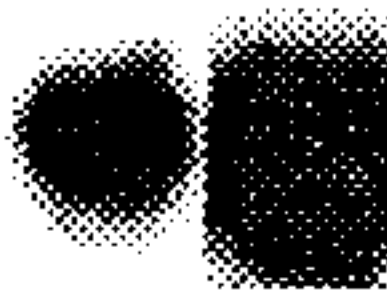
- +



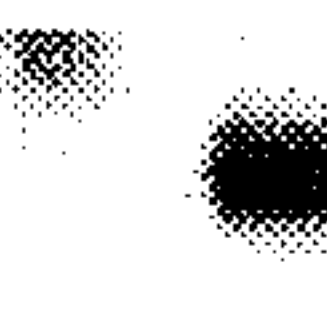
mda-4



mda-5



mda-6



mda-7



mda-8



GAPDH